

Fall 12-2016

Functional Significance of Branch Points in Mirtrons

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FUNCTIONAL SIGNIFICANCE OF BRANCH POINTS IN MIRTRONS

The University of Southern Mississippi

Functional Significance of Branch Points in Mirtrons

By

Britton A. Strickland

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
In Partial Fulfillment
Of the Requirements for the Degree of
Bachelor of Science
In the Department of Biological Sciences

December 2016

FUNCTIONAL SIGNIFICANCE OF BRANCH POINTS IN MIRTRONS

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Abstract

MicroRNAs are a heterogeneous group of small regulatory RNAs generated by many pathways. Mirtrons (miR) are a class of microRNAs produced by splicing, and some mirtrons contain a 3' tail located downstream from the self-complementary hairpin. During RNA splicing, a loop-like “lariat” intermediate structure is created when the 5' end of the RNA is attached to an adenine called the branch point. The goal of this project is to uncover the contribution of branch point location to the processing of tailed mirtrons into functional gene regulators. This project approaches this issue from two directions. First, branch points were identified by generating all possible lariat signatures from specific intronic samples using LaSSO and aligning the signatures to a genome-wide *Drosophila* RNA-sequence library using Bowtie2. Second, a polymerase chain reaction (PCR) based branch point mapping strategy was used to experimentally isolate, mutate, and verify the branch point location of miR-1017. Additionally, a synthetic miR-1017 mutant was cloned without any adenine nucleotides in the 3' tail. We examined the mutation's effects on *Drosophila* S2 cells with transfection and Northern analysis. Northern analysis showed that the mutated branch point actually increased the production of mature mirtrons and removing the branch point abrogated expression entirely, indicating that mirtron maturation may be very dependent on branch point and its location.

Keywords: microRNAs, miR-1017, lariat, branch point, *Drosophila*

Acknowledgments

I would like to thank my thesis advisor, Alex Flynt, for teaching me all that I know and for having the patience to put up with the learning process. This project would have been a complete failure without his guidance. I would also like to also thank Mosharrof Mondal and Matthew de Cruz for helping out with all experiments when time got the best of me, and for answering all the stupid questions that I was too afraid to ask anyone else. I could not have asked for better or more fun lab mates. The three years in the lab have provided me with a wealth of knowledge, and for that I am forever grateful.

I would like to thank the Honors College at USM for giving me inspiration and motivation to press on for this thesis. I would also like to thank Mississippi INBRE for providing me with lab facilities and funding, as well as plenty of opportunities to present my research. I would also like to thank the National Institute of Health for their funding through the Institutional Development Award (IDeA) under grant number P20GM103476.

Lastly and certainly most importantly, I want to thank my bride-to-be, Maribeth, for the incredible love and support throughout everything. Thank you for always patiently waiting on me when my experiments took longer than expected and for listening to me ramble on about microRNAs. Much love, forever and always.

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List of Abbreviations

Ago	Argonaute protein
cDNA	Copy DNA
miRNA	MicroRNA
mRNA	Messenger RNA
mutBP	Mutated branch point
noBP	Lacking any adenine residues in the 3' tail
PCR	Polymerase chain reaction
pre-mRNA	Primary RNA transcript containing both introns and exons
wt	Wild-type miR-1017

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Chapter 1: Introduction

MicroRNAs (miRNAs) are a heterogeneous group of short, ~22 nucleotide regulatory RNAs formed by a variety of pathways (Kim and Kim, 2007), as seen in Figure 1. MiRNAs are found in a variety of species, from plants to mammals and can cause detectable changes—either beneficial or harmful—to morphology, physiology, and even behavior (Rhoades et al, 2002; Berezikov et al, 2007; Flynt and Lai, 2008). MiRNAs in eukaryotes can target thousands of genes to inhibit protein translation. Research into miRNA regulation is critical to understanding the biogenesis and processes of these genetic “switches” is vital to understanding the biology of multicellular organisms, from embryonic development to brain activity (Flynt and Lai, 2008).

A specific class of miRNAs called mirtrons are short, intron-derived hairpins that bypass Drosha processing and are instead produced by splicing and lariat debranching via spliceosome (Martin et al, 2009), as seen in Figure 2. Immature mirtrons are folded into

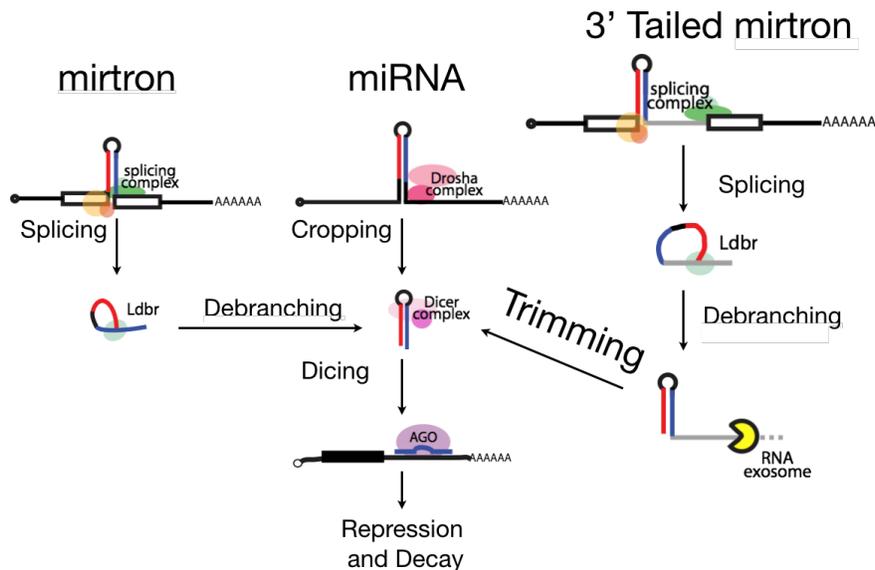


Figure 1: miRNA Biogenesis Pathways. miRNAs, mirtrons, and 3' tailed mirtrons are all formed through different pathways, but all undergo Dicer processing and Argonaut implementation to repress gene expression.

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intermediate fragments called lariats, which is when 5' end of the intron (always GU) is cut from the pre-mRNA and is covalently attached to a site called the branch point. After lariat debranching, mirtrons forms hairpins, which is processed by Dicer and transported by Argonaute (Ago) proteins to target mRNAs (Flynt et al, 2010). After they are processed, these small RNA products trigger repression and decay in target transcripts (Okamura et al., 2007). Production of mirtrons is well understood; however, the location of branch points in mirtrons have yet to be studied or identified. While the nature of branch point interaction is recognized, whether or not the 3' guanine interacts with a specifically located adenine within the intron is unknown. The goal of this project is to uncover any significant contribution of branch point location to the processing efficiency of mirtrons into functional gene regulators.

miRNAs are found in multiple species, and they make up nearly 600 genes in the

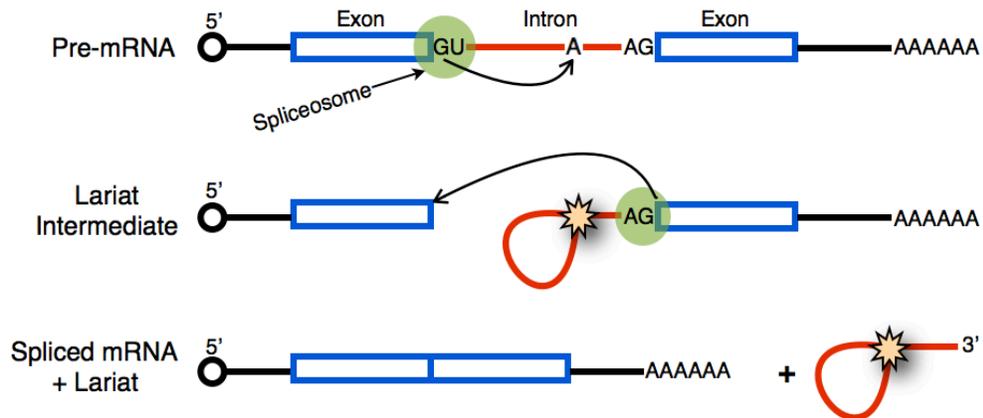


Figure 2. mRNA Splicing and Lariat Formation. Mirtrons bypass Drosha cropping, and introns are spliced out with a spliceosome complex. The result is a lariat formation when the 5' end of the intron (guanine) is covalently attached to the intron at the branch point (adenine). The lariat is eventually debranched and processed by Dicer to become a mature mirtron.

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human genome. Their regulatory functions are extremely efficient and essential to gene regulation (Martin et al., 2009). Understanding the biogenesis, structure, and function of miRNAs has been at the forefront of genetic research for quite some time. Uncovering the details of miRNA biogenesis and the functional significance of branch points in mirtrons will provide essential information on how organisms regulate gene expression, yielding direct implications for biomedical research.

This project analyzed sequence elements, specifically branch points, in miR-1017, a highly-expressed and conserved tailed mirtron in *Drosophila* (Flynt et al, 2010), in order to find any significant contributions of sequences to the processing of the gene regulator. Since most introns are usually degraded and mirtrons are not, miR-1017 must contain some properties that allow conservation and processing potential. The formation of lariats and the conservation of branch point interactions suggest that the sequence and structure of the lariat intermediates are a vital part to mirtron biogenesis. Therefore, this project will begin to analyze and alter these lariat sequence elements in order to uncover the significance of branch points in mirtron biogenesis. First, computational algorithms identified all possible lariat signatures from specific intronic samples to gather any possible lariat formation in the intronic sequences. These theoretical signatures were compared to an experimental *Drosophila* genome-wide miRNA-sequence to find lariat formations exist in specific species. A synthetic DNA sequence was designed where all possible branch points (adenine nucleotides) in the tail were removed, thus severely altering branch point formation. By placing the mutated branch point back into *Drosophila* cells and examining both the mature and immature mirtron expression using Northern analysis, we observed the effects of alternate branch points on the organism and

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made conclusions regarding the branch point's role in gene regulation and organismal viability. The results from this experiment bring light to an unexplored area of biological regulatory systems and provide crucial information to miRNA research throughout all species.

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Chapter 2: Literature Review

miRNA Pathways of Development and Regulation

miRNAs are short, ~22 nucleotide regulatory RNAs formed by a variety of pathways (Kim and Kim, 2007). miRNAs are key gene regulators that target mRNA before translation and complementary bind to specific regions of mRNA to signal repression and decay. Studies regarding miRNA regulation is an emerging field of research that has many aspects yet to be uncovered (for more information, see Almeida, Reis, and Calin, 2011).

The miRNA seed, a small 7 nucleotide sequence complimentary to miRNA positions 2 to 8, determine the target genes that the miRNA will repress (Martin et al, 2009). During biogenesis, canonical miRNAs form a hairpin shape via complementary base pairing of nucleotides, and the excess intronic fragment is cleaved from the pre-miRNA via RNaseIII enzymes Droscha and Pasha. (Okamura et al, 2007). A 2009 study by Martin et al examined DNA that was coded to bypass Pasha (and, subsequently, terminated Dicer processing), and they found that another class of miRNAs called mirtrons were produced instead. Unlike traditional miRNA biogenesis pathways, mirtrons bypass Droscha cleavage via spliceosome, and the pathway generates a lariat intermediate where the 5' end covalently attaches back onto itself. After lariat debranching, the mirtron folds into the hairpin shape that is cut and modified by Dicer and transported by Argonaute proteins. (Flynt et al, 2010). The construction of mirtrons is well identified; however, the identification of the mirtrons is still dependent on the computational identification of splice sites. Still, there is no evidence of patterns in

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mirtron splice sites (Westholm and Lai, 2011). After they are processed, these small RNA products signify repression and decay in target transcripts (Okamura et al., 2007).

Deep Sequencing and miRNA

Bioinformatics has made miRNA research easier by providing computational examination and analysis. miRNA research benefits from deep sequencing, a large-scale data analysis method developed for the interpretation of broad-spectrum samples of total RNA from an organism (Czerniecki and Wołczyński, 2011). Years of research and sequencing results have generated rich gene databases for short RNAs and has uncovered diverse classes of mirtrons that would have not been found through bioinformatic computational predictions alone. In *Drosophila*, the compilation of these large sequencing databases is one of the standard techniques for determining small RNA expression, regulation patterns, and biogenesis. The Berezikov et al (2011) study compiled 187 *Drosophila* small RNA data sets to create a rich database that included genetic information from nearly every piece of the fly: from biochemical pathways to specific body parts. From the results, the study found even more regulatory functions of miRNA, as well as identified some editing events for mature miRNAs that cause changes in target specificity and Ago sorting (2011). Furthermore, the information uncovered from these datasets and related research can be directly translated into animal and human studies (Kozomara and Griffiths-Jones, 2010).

Genome databases contain millions of base pairs that are a challenge to interpret. However, with a variety of computational algorithms, high-throughput sequencing results can be picked apart and sorted based on the needed information. For miRNAs, a memory-efficient software called Bowtie2 can align and re-sequence short DNA sequences from

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large mammalian genomes at high rates (Langmead et al, 2014). This is especially useful because it can find the desired DNA fragment inside the genome in order to determine presence and location. For research with the hairpin formation, another useful program is laSSO, which can generate constructs for every possible lariat formation (Bitton, 2014). This can gather intronic sequences and any possible mutation or formation. This data can then be aligned with RNA-sequencing data via Bowtie2 to see if the lariat formations exist in specific species. Specific RNA-sequencing databases have been made publically available, such as the *Drosophila* circular RNA library published by Ashwal-Fuss et al (2014). Many online genome databases are readily accessible, such as FlyBase. FlyBase has been crucial to *Drosophila* research, and it contains a variety of annotated genomes from different species (dos Santos et al, 2015).

miRNA in Mammals

miRNAs were first identified in *C. elegans* and have been extensively studied, especially mirtrons, in *Drosophila* species (Lee et al, 1993; Okamura et al, 2007). Now that the knowledge of miRNAs and their function has been explored, many researchers have begun to look for application in mammals. Using computational algorithms based on published genomes, ~22 nucleotide RNAs, identified as short hairpin introns and well-conserved mirtron models, were found in the RNA libraries of humans, macaques, chimpanzees, rats, and mice. In search of “newly evolved” mirtrons, an additional 16 primate-specific mirtrons were found in the human and macaque brain, as well as 50 additional candidates based on one or two cloned models (Berezikov et al., 2007). From these results, the aforementioned study shows a larger number of primates mirtrons than fly or worm mirtrons, revealing a large source of gene regulation in mammals.

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Surveillance of these gene regulators could lead to the prevention or treatment of many detrimental diseases due to the fact that these regulators are essential for biological development. For example, Flynt and Lai found that miRNAs were essential in muscle development in Texel sheep, and the over-expression of miRNAs could cause an excessive repression of the muscular protein myostatin in the sheep, which causes muscle overgrowth (2008). To the opposite effect, the over-expression of certain miRNAs in humans has been found to enhance cell proliferation in lung cancers (Hayashita et al 2005).

miRNAs make up nearly 600 genes in the human genome, and their regulatory functions are extremely efficient (Martin et al., 2009). In 2012, a study was conducted on animal and human genomes, and hundreds of mouse and human mirtrons were discovered (Ladewig et al., 2012). As more evidence of miRNAs is unveiled, the scientific community will further understand how our bodies regulate gene expression, which is especially useful when these regulators go awry. It is speculated that in mammalian miRNA, the deletion of miRNA genes could lead to cardiac, immune, neurological, and metabolic disorders (Hansen et al 2016, Mang et al 2015). Even so, over-expression of miRNAs could be detrimental (Rommer et al 2013, Yan et al 2008, Chen et al 2015).

miRNA Conservation

High-throughput sequencing results have concluded that many mirtrons were created fairly recently through evolution (Okamura et al, 2007). These mirtrons have been found throughout many species, with many mirtrons being highly conserved across evolution. miR-1017 is a highly expressed 3' tailed mirtron found across 12 sequenced

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Drosophila species, and it is the only highly-conserved tailed mirtron in the species (Flynt et al 2010). Small RNA sequencing is one of the most important technologies used to identify newly evolved miRNAs. The Chung et al study found that newly evolved mirtrons, even at low-expression levels, can be confirmed by mapping the small RNA reads to the available genomes to determine evolutionary relationships. The study also used the data, along with other published mirtron sequences, to develop a highly specific computer model that identifies genuine mirtrons (2011).

miRNA in Lab Studies

Lab-based approaches utilize a variety of options for isolating and examining miRNAs. Isolation can be performed by generating copy DNA (cDNA) from RNA via reverse transcription and replicating specific sequences via PCR (Ashwal-Fuss et al, 2014). To understand how crucial splicing is to mirtron biogenesis, small mutations can be introduced to disrupt the splice sites. These mutations may or may not alter the function and maturation of the mirtrons, and the effect can be studied. Introns can be examined and studied in detail using specific primers and PCR to determine splice sites. In order to examine if mutated introns have any effect on cells, HeLa can be transfected with a variety of mirtrons, primers, and antibodies. RNA can be extracted, and Northern analysis will be used to verify mirtronic production and expression in the cell (Ladewig et al., 2012).

Chapter 3: Methods

Computational Identification and Analysis

LaSSO, developed by Bitton, computed all possible lariat formations from the intron that codes for miR-1017 (2014). This program took the 5' guanine and paired it with all adenines within miR-1017. Bowtie2, developed by Langmead et al (2014), was used to align these signatures to short cDNA sequences from a publicly-available *Drosophila* circular RNA library in order to identify any lariats that exist in nature. The circular RNA library was constructed by Ashwal-Fuss et al with the TruSeq RNA sample preparation kit from Illumina using total RNA from fly heads with ribosomal and other linear RNA removed via RNaseP (2014). These alignments were visualized by subsequent alignment to the known miR-1017 sequence using the Clustal Omega (Sievers et al, 2011) computer model in order to map the branch point. This process is illustrated in the pipeline in Figure 3.

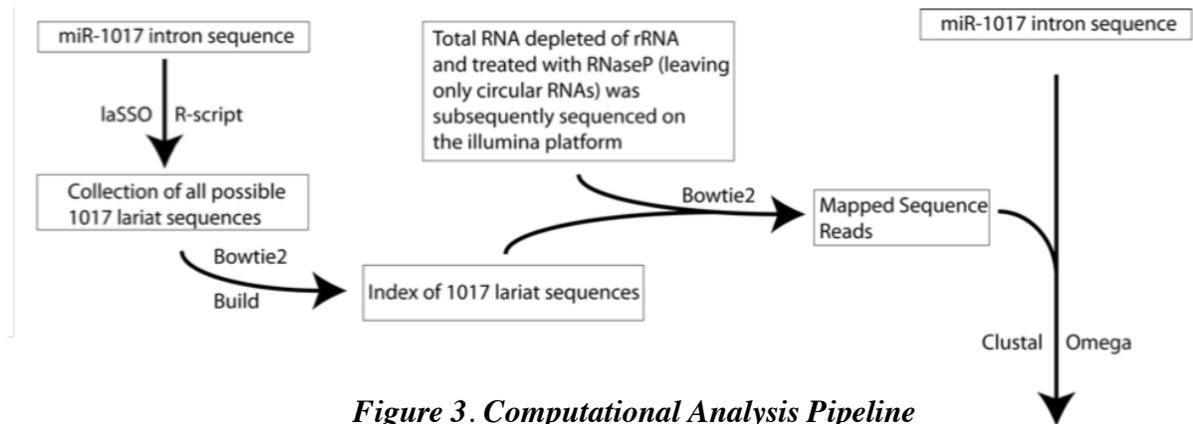


Figure 3. Computational Analysis Pipeline

Site-Directed Mutagenesis

Once the branch point was located computationally, miR-1017 was mutated using a polymerase chain reaction (PCR) based branch point mapping strategy in order to

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observe the importance (if any) of branch point location. The branch point was mutated (mutBP) using SOEing PCR (gene splicing by overlap extension) strategy developed by Horton (1995), as well as custom designed plasmids specific to miR-1017 intended to omit the sequence of base pairs surrounding the branch point identified using the computational methods. This protocol (figure 4) required amplifying two separate miR-1017 PCR products, one amplified using pUastdsRedF/bpMutantAS (PCR1) and the other amplified using pUastdsRedR/bpMutantS (PCR2).

pUastdsRedR: ACAGAAGTAAGGTTTCCTTCACAAAGATCC
 bpMutantS: CTACCCAAACTCATCCcccgcctttctccct
 pUastdsRedF: ACCATCGTGGAGCAGTACGAG
 bpMutantAS: aggggagaaagggcgggGGATGAGTTTGGGTAG

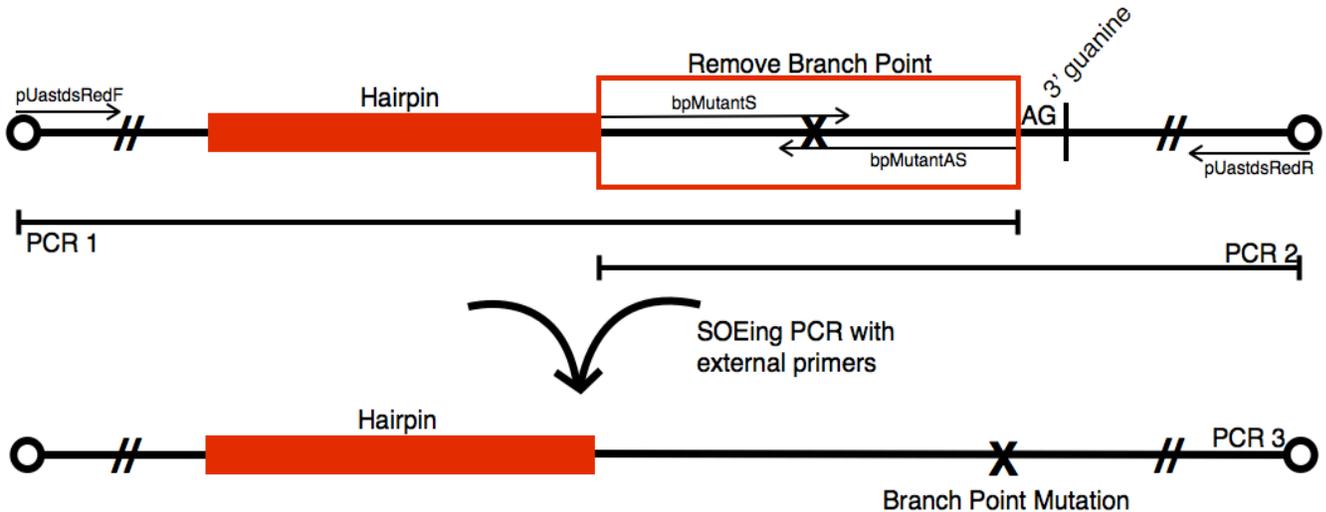


Figure 4. SOEing PCR Schematic. PCR1 and PCR2 were created with specific primers with the intention of omitting the known branch point. Both PCRs were sewn together (PCR3) via Slice extract to implement the branch point mutation. The effects of the branch point mutation were then examined through Northern analysis.

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PCR 1 and 2 were each run on a 1% low melt agarose gel. Each band was cut from the gel and melted at 65°C. 3µL of each melted gel slice were used as the template for the SOEing PCR (PCR3), which “sewed” PCR 1 and 2 together using pUastdsRedF and pUastdsRedR as the primers. PCR3 was then run on a 2% agarose gel and extracted using GeneJET Gel Extraction Kit. This protocol includes cutting the band from the gel, melting the gel slice in a 1:1 ratio of Binding Buffer, and washing the melted gel through a GeneJET purification column.

PCR3 was inserted into a dsRed1017 vector, a plasmid containing a red, fluorescent gene. The vector was prepared via restriction digestion with Fast Digest XhoI and NotI from ThermoScientific© and extraction from a 2% agarose gel. MutBP was inserted into the dsRed1017 vector using Slice extract (Zhang, Werling, and Edelman, 2012) using a 1:4 ratio of vector to insert and incubating at 37°C for 2 hours. MutBP:vector complex was then transformed for amplification (see *Molecular Cloning*). The amplified DNA product was then sequenced and analyzed to help determine any patterns in mirtron branch points. Northern analysis were used to verify mature mirtron production and the expression of the mutant in the cell (see *Examining Effects on Drosophila S2 Cells using Northern analysis*).

Molecular Cloning

DH5α competent cells were transformed using ~100ng of plasmid DNA and plated on LB-ampicillin agar plates with IPTG and Bluogal. Plates were incubated at 37°C for 16 hours. White colonies were selected and incubated in liquid LB with ampicillin for another 16 hours at 37°C. DNA was extracted from the cell culture using

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ThermoScientific© GeneJet Plasmid Miniprep Kit, and the concentration was measured with a spectrophotometer.

Branch Point Removal

A synthetic miR-1017 plasmid containing no adenine residues in the 3' tail (noBP) was ordered from 1DT Technologies and noBP was inserted into the cut dsRed1017 vector using Slice extract (see *Site-Directed Mutagenesis*). NoBP plasmid was cloned for amplification via transformation (see *Molecular Cloning*) and sequenced to verify the insertion. Northern analysis was used to examine the effects of branch point removal on *Drosophila* S2 cells. In order to determine if any splicing occurred, noBP was reverse transcribed using ThermoScientific© RevertAid Reverse Transcriptase Kit, which uses oligo(dT)₁₈ nucleotides, dNTP, RNase inhibitor, and reverse transcriptase to make RNA to copy DNA (cDNA). cDNA was amplified via PCR to examine whether or not splicing occurred. The PCR sample was run on a 1% agarose gel, and the band was extracted and sequenced with pUastdsRedF.

Examining Effects on *Drosophila* S2 Cells via Northern Analysis

Wild-type dsRed1017, mutBP, and noBP were transfected into *Drosophila* S2 cells using the TranIT®-Insect Transfection Reagent from Mirus Bio LLC. Each plasmid DNA was inserted into a dsRed1017 vector because the dsRed construct allowed ectopic expression of miR-1017, and the successfully transfected cells would illuminate red under fluorescent light. For the transfection protocol, 2.5µg of each plasmid DNA sample was mixed with ubGAL4 prior to transfection in order to signal amplification by the UAS sites in the dsRed plasmid. For the transfection protocol, 2.5mL of $\sim 4.0 \times 10^5$ cells/mL *Drosophila* S2 cells were aseptically plated in 3 separate wells of a 6-well plate. The

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plasmid DNA, mixed with ubGAL4, and 5µl of room-temperature TransIT-Insect Reagent were added to 250µl of Grace's Insect Basal Medium in a sterile tube and mixed gently. The mix was incubated at room temperature for 30 minutes. The TransIT-Insect Reagent:DNA mix was slowly added dropwise to each well and gently mixed. The cells were incubated for 72 hours and imaged using fluorescent microscopy. RNA was extracted from each culture using TRI Reagent® from MRC.

Northern analysis was performed using techniques adapted from Flynt et al (2010). 20ng of each RNA was precipitated with 2.5x volume of 200 proof ethanol and a 1/10 volume of 3M NaOAc and suspended in 5µL of 2X Sample Buffer (Ambion). The samples were run on a 12% sequence gel at 250V/30mA for 3 hours. The gel, stained with ethidium bromide, was then transferred onto a nylon membrane in 0.5X TBE for 1 hour at 4°C and crosslinked with the UV-crosslinker. The membrane was dried at 80°C for 30 minutes and prehybridized with hybridization buffer at 45°C. The membrane was then labeled with a radioactive Gamma-ATP probe and washed with a Roche spin column, developed on phosphorus screens, and imaged on the Typhoon Fla 7000.

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Chapter 4: Results

Computational Identification and Analysis

Out of the several thousand possible lariat branch point locations in miR-1017 generated by laSSO, 2 reads were found to be present after comparing the possible lariat formations to the *Drosophila* circular RNA genome database. This branching pattern can be seen in Figure 5.

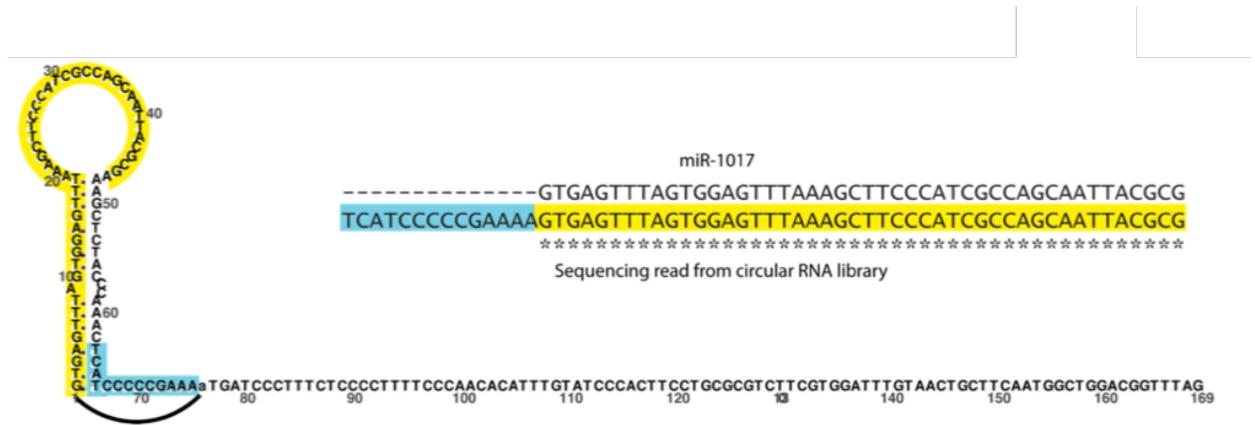


Figure 5. Clustal Omega. Sequencing reads from the circular RNA library shows branching pattern between adenine and guanine. This image was constructed by Clustal Omega.

Site-Directed Mutagenesis

After the SOEing PCR series was conducted, the product was sequenced to find the branch point location removed (figure 6a). Splicing still occurred in S2 cells based on the results from Northern analysis, but the location of the new branch point is something to be further studied. Transfected cells illuminated red to indicate that the spliced product was being expressed (figure 6b).

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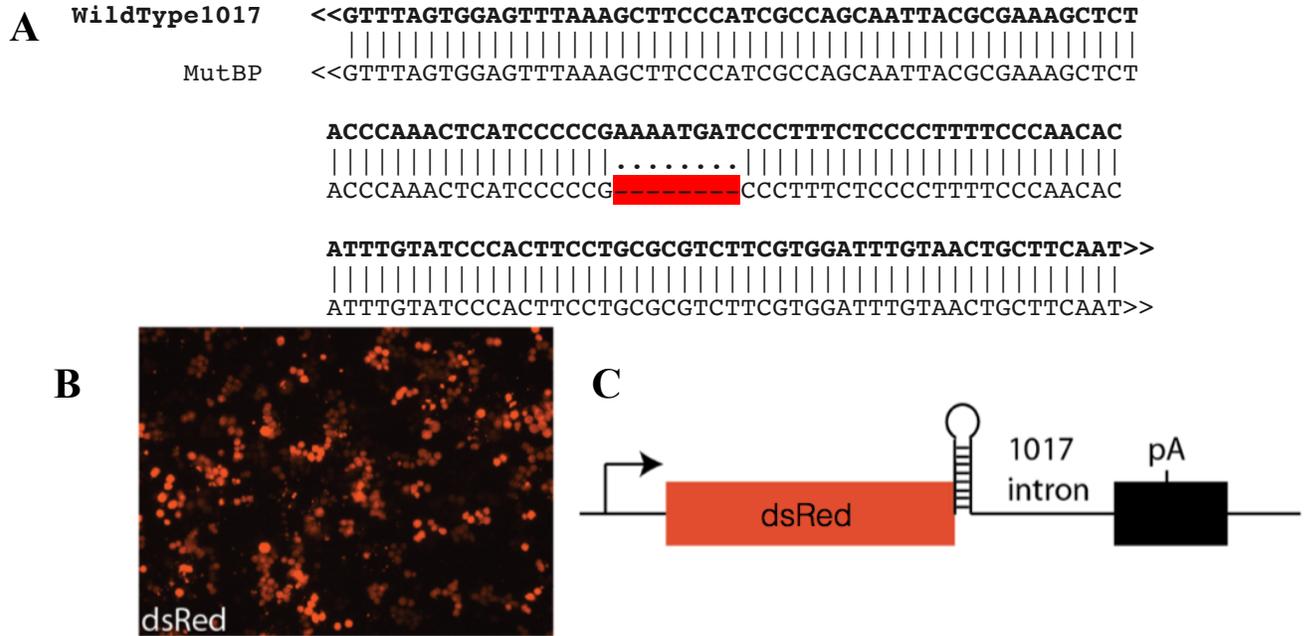


Figure 6. Mutated Branch Point Analysis. A) WildType 1017 vs mutBP. The highlighted region indicates the deleted branch point region within the intron. B) Cells that positively express the plasmid illuminate red under florescent light. C) Cells were transfected with a plasmid containing a red, florescent protein (dsRed) upstream from the hairpin and mutBP at the location of the 1017 intron.

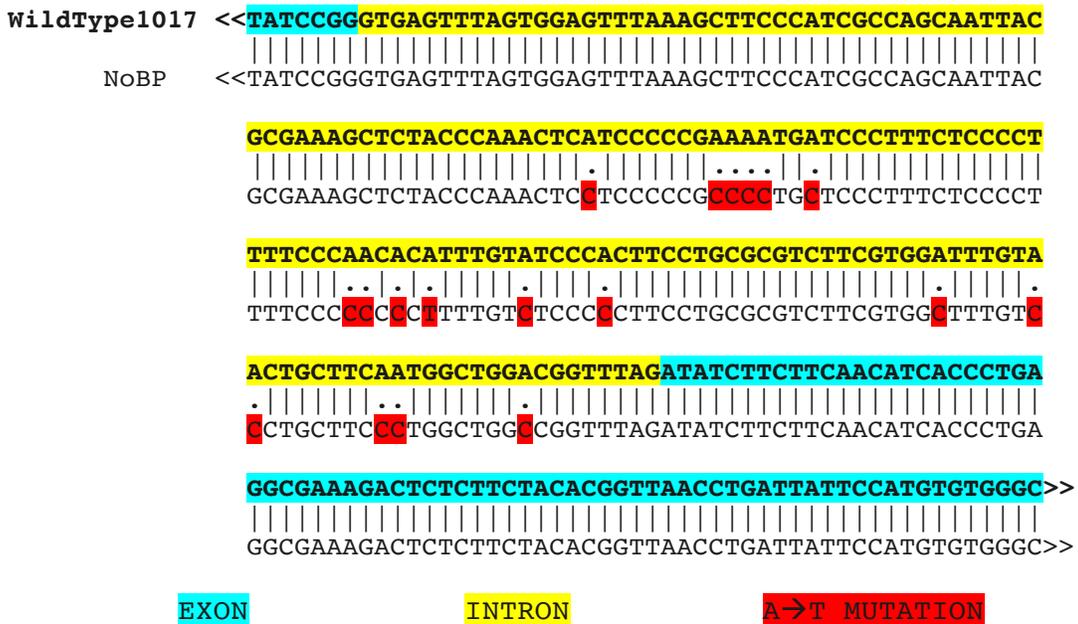


Figure 7. Branch Point Removal. Clustal Omega nucleotide alignment of WildType 1017 vs noBP. Highlighted nucleotides indicate where adenine was replaced by thymine within the intron (yellow).

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Branch Point Removal

The synthetic mutBP without any adenine residues (noBP) was successfully amplified in competent cells, and the sequencing results in Figure 7 shows the replacement of individual base pairs. While processing into mature mirtrons was not evident based on Northern analysis (see *Results: Expression Levels of Mutated Constructs*), expression and splicing of the noBP construct were observed based on the sequencing results (Figure 9). The intron was successfully spliced out at both the 5'GU and the 3'AG, as indicated by the underlined sequencing results.

Expression Levels of Mutated Constructs

After the Northern analysis was performed, the differences in expression due to the effects of branch point location were evident. In the wild type 1017 construct, a miniscule amount of mature mirtrons were produced (at 22nt). In the mutBP construct, a significantly larger amount of mature mirtrons were produced. In the noBP construct no immature or mature mirtrons were produced (Figure 8).

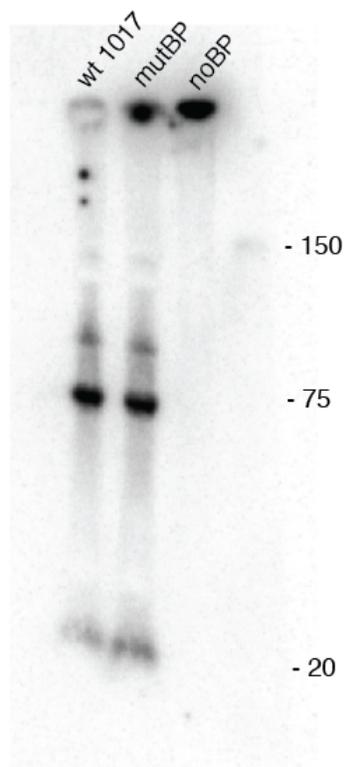


Figure 8. Northern Analysis. Northern blotting was performed on both the wt (lane 1), mutBP (lane 2), and noBP (lane 3). For the wt, only a few of the immature mirtrons (band at ~70nt) were processed into mature mirtrons (band at ~22nt). For mutBP, a significantly larger amount of mature mirtrons were produced. For noBP, no mature or immature mirtrons were produced, indicating that the spliced product was not further processed.

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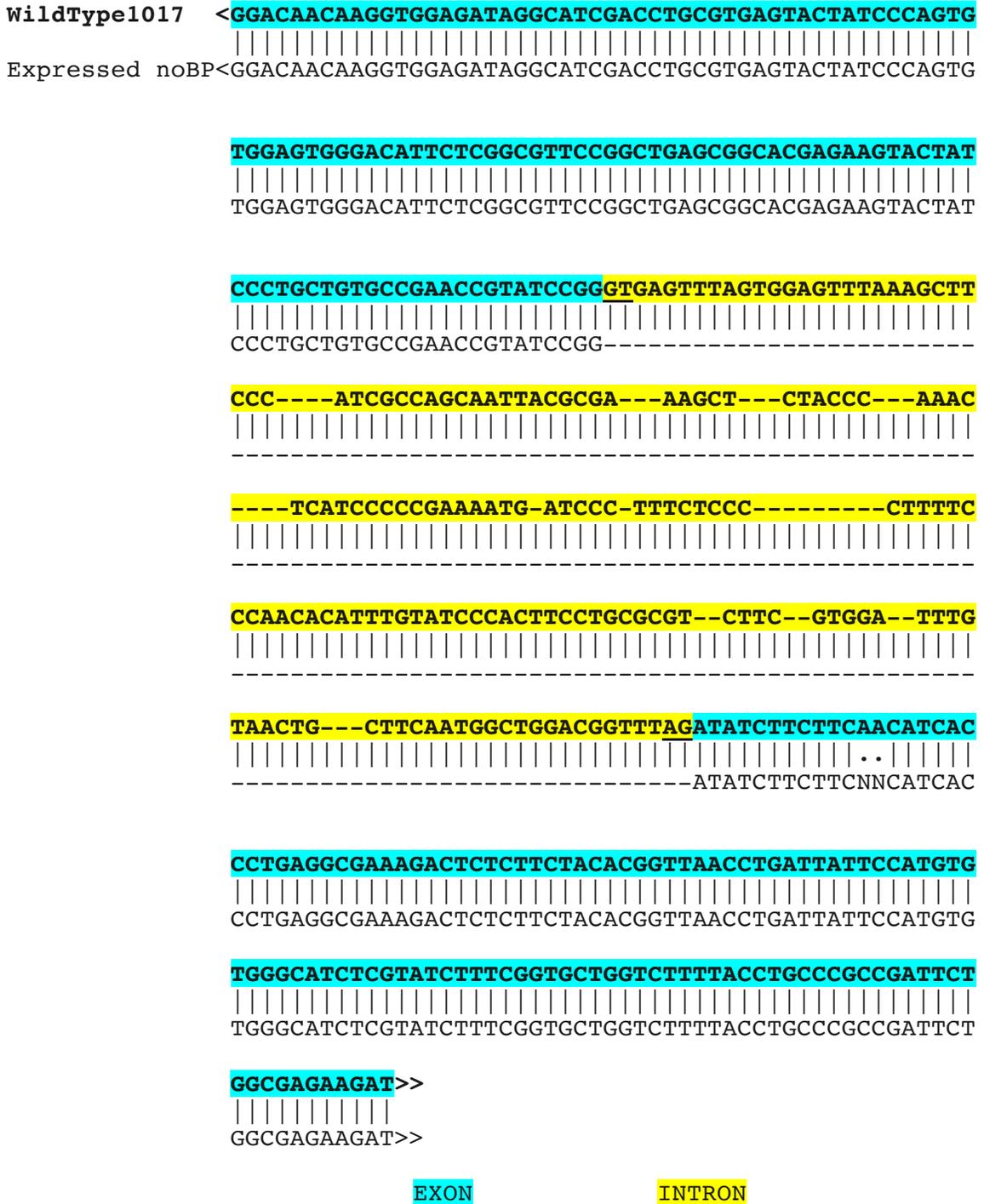


Figure 9. NoBP Mapping. Northern analysis showed that no mature or immature mirtrons were produced by the cells from the noBP construct; in order to see if the cell expressed the construct, the noBP RNA was reverse transcribed, amplified via PCR, and sequenced. The results show that the noBP construct was expressed in the cell because the intron (yellow) was spliced out at the “AG” 5’ splice site and the 3’GU site. However, branch point attachment occurred outside of the 3’ tail, leading to no lariat intermediate and, consequentially, no mature mirtrons.

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Chapter 5: Discussion and Conclusions

The location of branch points in tailed mirtrons has been acknowledged by earlier research, but the significance of the odd base pair interaction and the specific location has been unknown. By examining some of the conserved features in tailed mirtron production, such as the branch point, a lot can be uncovered about the features of lariat formation and possible unknown patterns. Computational analysis initially suggested that the 5' guanine attached to one adenine at a specific location within the 3' tail of the intron due to the fact that only 2 of the few thousand potential lariat branch points were found in the *Drosophila* RNA-seq database. By mutating this branch point, our analysis showed that mature tailed mirtron production was significantly altered when the base pair location was altered. Northern analysis showed that the removing a small section of branch points did not hinder mature tailed mirtron production but actually increased the production, indicating that the branch point mutation aids in evasion of miRNA degradation in order to produce more mature tailed mirtrons. Additionally, we determined the importance of a branch point within the 3' tail by removing the branch point possibilities (adenine) entirely with the aid of a synthetic miR-1017 construct. When the branch point was removed entirely, splicing of the intron occurred based on sequencing results, but Northern analysis showed both mature and immature tailed mirtrons were not produced. This indicates that the lariat intermediate must form a branch point within the 3' tail in order for Dicer processing to occur, and, furthermore, the location of the branch point has a strong impact on the amount produced. Overall, these results suggest that the sequence elements in miR-1017 contribute to the regulation of the mirtron itself, which helps explain mirtron conservation and miRNA biogenesis.

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