Aret: a novel regulator of alternative splicing in the flight muscle transcripts in Drosophila melanogaster

Sandy T. Oas
Sandy Thi Oas
Candidate

Biology
Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

Richard M. Cripps, Chairperson

Stephen A. Stricker

Rebecca S. Hartley
ARET: A NOVEL REGULATOR OF ALTERNATIVE SPlicing IN FLIGHT MUSCLE TRANSCRIPTS IN DROSOPHILA MELANOGASTER

By

Sandy Thi Oas

B.S., Biology, University of New Mexico, 2010

THESIS

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

Biology

The University of New Mexico
Albuquerque, New Mexico

December 2014
Acknowledgements

Thank you to Richard Cripps, D. Phil., for his mentorship and support throughout my career in the Cripps Laboratory. His guidance will profit me in my future endeavors. I would like to thank the guidance and leadership provided by Dr. Anton L. Bryantsev during this project. I will carry his innovative thinking throughout my career.

I would like to acknowledge Dr. Rebecca Hartley and Dr. Steve Stricker as members of my thesis committee and their input during this project and Dr. Stephen Jett for his contribution to the EM pictures. Special thanks to the funding agency, the National Institute of Health for supporting my research.

I would like to sincerely thank Tonya Brunetti for her continued support and assistance during this project. Thank you for taking the time to share your pragmatic advice and knowledge to me, and with this help, I know I have become a more solid scientist. I would like to thank Antonio Banuelos for his counsel and support during my graduate career. Additionally, I would like to thank Kathryn Ryan, Maria Chechenova, TyAnna Lovato, and Ashley DeAguero for their assistance and invaluable advice during this project. To the members of the Cripps laboratory and my family, I would like to extend many thanks for their encouraging words and support.

Finally, I would like to thank Vance Oas for his continued support and words of encouragement. He continuously gave me reassurance and confidence during my graduate career. I know without him as my foundation, I would not have succeeded.
ARET: A NOVEL REGULATOR OF ALTERNATIVE SPLICING IN FLIGHT MUSCLE TRANSCRIPTS IN DROSOPHILA MELANOGASTER

By

Sandy Thi Oas

B.S., Biology, University of New Mexico, 2010
M.S., Biology, University of New Mexico, 2014

ABSTRACT

Drosophila melanogaster has been used as a model organism for understanding muscle development. Drosophila flight and jump muscles are distinct functionally and biochemically. This distinction is due to differentially expressed genes and differentially spliced mRNA transcripts. The exact mechanism of alternative splicing in somatic muscles is not well characterized.

Aret was previously shown to be a transcriptional repressor, and has been implicated in splicing regulation based upon literature analysis and preliminary work. This study aims to define the regulatory role of Aret and the impact of alternative splicing on determining muscle diversification and fiber choice. We indicate the importance of Aret in determining flight muscle structure and function. Without this protein, the animal exhibits molecular and structural changes within the flight muscles.

The CELF family proteins are mammalian orthologs of Aret and are involved in regulation of alternative splicing, which suggests the function is evolutionarily conserved between Drosophila and mammals. An incorrect dosage of CELF leads to cardiomyopathies and myotonic dystrophies reinforcing the functional relevance of this family of proteins and Aret.
# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ VI

**ARET: A NOVEL REGULATOR OF ALTERNATIVE SPLICING IN FLIGHT MUSCLE TRANSCRIPTS IN *DROSOPHILA MELANOGASTER*** ........................ 1

**ABSTRACT** .................................................................................................................. 1

**INTRODUCTION** ....................................................................................................... 2

**METHODS** ................................................................................................................ 6

- **FLIES** ....................................................................................................................... 6
- **TISSUE ANALYSIS** .................................................................................................... 8
- **CELL CULTURE** ........................................................................................................ 9
- **EXPRESSION ANALYSIS** ........................................................................................ 10
- **MOLECULAR CLONING** .......................................................................................... 11
- **BIOINFORMATICS** ................................................................................................... 12
- **STATISTICAL ANALYSIS** ......................................................................................... 13

**RESULTS** .................................................................................................................. 13

- **FUNCTIONAL TEST OF THE ARET KNOCKDOWN** .................................................. 13
- **VALIDATION OF ARET KNOCKDOWN** .................................................................... 14
- **IDENTIFICATION OF ARET AS A REGULATOR OF ALTERNATIVE SPLICING IN FLIGHT MUSCLES USING RT-PCR ANALYSIS** .................................................. 15
- **ARET KNOCKDOWN RETAINS FLIGHT MUSCLE SPECIFIC IDENTITY** .................. 18
- **ARET LOCALIZATION IN THE NUCLEI OF FLIGHT MUSCLES AND ITS PRECURSORS** .................................................................................................................. 19
- **CHARACTERIZATION OF ARET EXPRESSION IN FLIGHT MUSCLE FOUNDER CELLS** .............................................................................................................. 21
- **IDENTIFICATION OF THE ISOFORM OF ARET IN THE FLIGHT MUSCLES** ................ 22
- **ARET KNOCKDOWNS EXHIBIT ULTRASTRUCTURAL CHANGES IN FLIGHT MUSCLE MORPHOLOGY** .................................................................................... 23
- **ARET IS SUFFICIENT IN THE JUMP MUSCLES AND IN TISSUE CULTURE CELLS TO ENFORCE FLIGHT MUSCLE SPECIFIC SPLICING** ................................................. 26
- **IDENTIFICATION OF RNA SEQUENCES FOR ARET-RNA INTERACTION** .............. 29
- **ARET IS FOUND WITHIN A TRANSCRIPTIONAL FRAMEWORK OF MUSCLE IDENTITY** ............................................................................................................ 32
- **SCHEMATIC OF ARET IN A TRANSCRIPTIONAL FRAMEWORK** ................................. 33

**DISCUSSION** .............................................................................................................. 34

**REFERENCES CITED** .................................................................................................. 38
LIST OF FIGURES

FIGURE 1: FUNCTIONAL TEST OF THE ARET KNOCKDOWN..................13
FIGURE 2: VERIFICATION OF THE ARET KNOCKDOWN..........................14
FIGURE 3: IDENTIFICATION OF ARET AS A REGULATOR OF ALternative SPLICING IN FLIGHT MUSCLES USING RT-PCR ANALYSIS ...............15
FIGURE 4: ARET KNOCKDOWN RETAINS FLIGHT MUSCLE SPECIFIC IDENTITY.................................................................18
FIGURE 5: ARET LOCALIZATION IN THE NUCLEI OF FLIGHT MUSCLES AND ITS PRECURSORS .................................................................19
FIGURE 6: CHARACTERIZATION OF ARET IN FLIGHT MUSCLE FOUNDER CELLS.........................................................................................21
FIGURE 7: IDENTIFYING THE ISOFORM OF ARET IN THE FLIGHT MUSCLES .........................................................................................22
FIGURE 8: ARET KNOCKDOWN EXHIBITS ULTRASTRUCTURAL CHANGES IN FLIGHT MUSCLE MORPHOLOGY ............................................23
FIGURE 9: ARET IS SUFFICIENT IN THE JUMP MUSCLES AND TISSUE CULTURE CELLS TO ENFORCE FLIGHT MUSCLE SPECIFIC SPLICING ..................................................................................................................26
FIGURE 10: IDENTIFICATION OF RNA SEQUENCES FOR ARET-RNA INTERACTION...........................................................................29
FIGURE 11: ARET IS FOUND WITHIN A TRANSCRIPTIONAL FRAMEWORK OF MUSCLE IDENTITY ................................................................32
FIGURE 12: SCHEMATIC OF TRANSCRIPTIONAL FRAMEWORK ...........33
ARET: A NOVEL REGULATOR OF ALTERNATIVE SPlicing IN FLIGHT MUSCLE TRANSCRIPTS IN DROSOPHila MElANOgASTER

ABSTRACT

We have used *Drosophila melanogaster* to study genetic mechanisms governing differentiation of adult somatic muscles into different fiber types. *Drosophila* flight and jump muscles are distinct functionally and biochemically due to differentially expressed genes and differentially spliced mRNA transcripts. It is thought that transcription factors are the main contributors to tissue specificity, but a potential role for alternative splicing needs to be further elucidated due to the growing number of genes being discovered for which the primary transcripts are alternatively spliced. Using comparative dataset analysis and literature search, we found that *aret* is differentially expressed across different muscle types and encodes a protein with RNA-binding domains. This study aims to determine if *aret* is a novel alternative splicing regulator in the adult fly musculature. Here, we show endogenous Aret protein is localized to the nuclei of the flight muscles, and absence of Aret results in flightless adults with ultrastructural changes of flight muscles. The removal of Aret within the flight muscles leads to a change in alternative splicing resulting in jump muscle patterned transcripts. This study indicates Aret works independently to induce flight muscle splicing patterns when ectopically expressed in the jump muscle and in tissue culture. The mammalian orthologs of Aret, the CELF proteins, are important regulators of splicing and are implicated in cardiomyopathies and muscular dystrophies reinforcing the functional importance of our results.

**Keywords:** *Drosophila*, Aret/Bruno, alternative splicing, muscle fiber type
INTRODUCTION

Somatic muscles of insects and vertebrates consist of different fiber types, evident on molecular, morphological, and physiological levels. In vertebrates, the different muscle types are known as fast- and slow-twitch muscle fibers (Schiaffino et al., 1970). The fly has a diversity of muscles within the thorax: indirect flight muscles (IFMs), jump muscles (TDT), and direct flight muscles. One feature that characterizes different types of muscles is the genes that are differentially expressed allowing for unique biological and physiological functions of the specific muscle type (Bernstein et al., 1993; Vigoreaux, 2001). Different muscles require different genes to be expressed to confer biochemical properties that allow for a diversity of functions. Hox cofactors, exd and hth, are transcription factor genes that are expressed in the flight muscle and have been characterized as muscle identity genes. Further analysis has shown that the transcription factor salm is downstream to exd/hth in this complex process of muscle specificity (Bryantsev et al., 2012). The goals of this study are to add to this transcriptional framework by understanding how muscle-specific alternative splicing can be controlled.

Alternative mRNA splicing is an important mechanism of gene regulation mechanism that allows for a large array of proteins to be expressed from only 32,000 genes in the human genome. Alternative splicing is involved in a wide range of biological processes from sex determination to apoptosis (Modrek and Lee, 2002). The process that decides which exon is included and which is excluded is regulated by proteins and RNA sequence elements. Alternative
mRNA splicing is a versatile process that integrates alternative transcripts into a large regulatory framework, allows for modulation of cellular responses to stimuli, and adds diversity within the biochemical repertoire found in cells. Regulated splicing is critical to human health; for example, when splicing of *Wilm’s Tumor Locus* is disrupted the result is a change in ratios of alternatively spliced proteins and a urogenital disease such as Denys-Drash Syndrome can arise (Lopez, 1998).

Transcription factors are thought to be the main contributors to tissue specificity. However, many cases have been identified in which tissue specific gene expression involves the usage of alternatively spliced genes to create a variety of protein isoforms. More than 2,500 transcription factors are found in humans, but less than 50 sequence specific alternative splicing regulators have been characterized (Chen and Manley, 2009). Presumably, many splicing regulators have not yet been discovered. Clearly this mechanism of splicing and how it is regulated needs to be understood in greater detail due to the growing number of genes that are showing alternative splicing (Chen and Manley 2009; Brietbart *et al.*, 1987).

The *Arrest (aret)* gene that is formally known as the Bruno protein shares 45% identity with the human protein, CELF2. Considering the distance of *Homo sapiens* from *Drosophila*, this percentage of conservation suggests that Aret performs an essential and conserved function (ensembl, Snee *et al.*, 2008). Many splicing regulators use recognition motifs (RRMs) to interact directly with mRNA. RRM are the most common RNA binding domains in various species of
plants, fungi, and animals. Aret contains three RNA RRMs that are conserved across species. These binding domains allow the protein to bind to RNA regulatory units. Having multiple RRMs indicates combinatorial binding leading to high specificity and affinity by increasing the number of contacts between RNA and the RRMs (Reveal et al., 2011). This further supports the view that the conservation of RRMs is important to many fundamental processes, such as mRNA stability and alternative splicing. The presence of RRMs within Bruno suggests a similar function of mRNA processing for this protein.

This study aims to identify the regulation and function of an alternative splicing regulator that can be added to the repertoire of known regulators. We aim to understand which factors contribute to muscle fate and specificity in muscle development during splicing, and how this process can lead to muscular diseases when disrupted, such as myotonic dystrophy 1 and 2, and cardiac myopathies (de Die-Smulders et al., 1998; Ladd et al., 2005).

CELF is the vertebrate ortholog of aret, and studies have defined a role for the CELF family in somatic and cardiac muscle gene expression. For example, Berger et al. 2011 illustrate the essential function of CELF proteins and their regulatory roles in skeletal muscle by creating a CELF dominant negative mutant restricted to the nucleus. In the mutant mice exhibiting a loss of CELF function in vivo, small changes in CELF activity alter muscle organization, size, and subtype including an increase in the proportion of slow twitch muscle fibers from inherently fast twitch muscles (Berger et al., 2011).
The heart expresses high levels of CUG-BP, ETR-3 and CELF4 (Ladd et al. 2001). Studies have shown CELF4 mediates *myotubularin-related protein 1* (*mtmr1*) alternative splicing. When this splice event is disrupted due to lack of CELF expression, cardiac hypertrophy occurs in 9-week old mice (Ladd et al. 2005a). In the cTNT gene, there is evidence of exon 5 inclusion when CUG-BP (CELF1) and ETR-3 (CELF2) proteins are present in the fetal heart. In the adult heart, these proteins are down-regulated and are not able to promote exon inclusion. This indicates coordinated modulation of CELF proteins during heart development (Ladd et al. 2005b).

Myotonic dystrophy is due to lack of exon 2 inclusion and dysregulation of exon 10 in *tau*, a family of microtubule associated proteins. When CELF2 is over-expressed in somatic muscles, mis-splicing of *tau* will occur. Dhaenens et al. 2011 indicate the gain of function of CELF2 causes improper splicing events for exons 2 and 10. That in turn results in myotonic dystrophy (2011).

Classic myotonic dystrophy 1 (DM1) has a worldwide prevalence of 1/20,000. It is inherited in an autosomal dominant manner and has a young age of onset at 20-30 years old with a high penetrance of nearly 100% by the age of 50 (Bird, 2013). 88% of DM1 patients survive to age 45 and 18% survive to age 65, whereas patients without the disorder show a rate of survival of 95% and 78%, respectively (de Die-Smulders et al., 1998). From these studies, the importance of proper dosages of splicing regulators for cardiac and skeletal muscle genes can be inferred. When splicing is not properly maintained in the
mRNA of these genes, the resulting phenotype is muscular dystrophies with lower survival rate.

However, it is not clear the extent of impact the CELF proteins have upon tissue-specific patterns of splicing. Here, we show endogenous Aret protein is localized to the nuclei of the flight muscles within the thorax, and absence of Aret results in flightless adults with ultrastructural changes in the flight muscles. This study indicates Aret works independently when ectopically expressed in the jump muscle and tissue culture. Finally, Aret’s alternative splicing function is mediated by potential binding to conserved intronic sequences in a model muscle gene.

This study will increase our understanding of Aret’s role in somatic muscle development. Due to the high conservation between CELF2 and Aret, Aret can be used to further identify novel regulatory pathways in *Drosophila* to uncover an evolutionarily conserved pathway for controlling muscle tissue diversification.

**METHODS**

*Flies*

Fly stocks were obtained from Bloomington *Drosophila* Stock Center or Vienna *Drosophila* RNAi Center (VDRC) and maintained on Fischer-Scientific Jazz Mix medium. The *Gal4/UAS* system was utilized during the RNAi knockdown experiments (Duffy, 2002; Sik Lee and Carthew, 2003). Equal numbers of virgin females (*Act88F-Gal4*) and males (knockdowns) were crossed and incubated at 25°C until white pupae formed. After white pupae formation, the crosses were placed in the 29°C to induce tissue-specific effects in the flight muscles of the developing pupa (Bryantsev *et al.* 2012). The following RNAi-
inducible fly lines were used in this study: 32948 (targeting Spf45, obtained from VDRC), 48237 and 41567 (aret, VDRC), 104334 (snf, VDRC), 105495 (mub, VDRC), 100805 (tra2, VDRC), 22186 (Rsf1, VDRC), 105135 (psi, VDRC), 2912 (bl, VDRC), 34637 (hth, BDSC), 100687 (exd, VDRC), and 101052 (salm, VDRC). Among the two lines tested, the line for UAS-aret IR, 48237, was most effective in our analysis. The progeny of the crosses were analyzed for flight ability. Flight testing was performed as previously described by measuring at least 20 flies per genotype exhibiting null/flightless, down flight, horizontal flight, or upward flight behavior using a flight chamber (Drummond et al., 1991). The animal was categorized by landing behavior where landing upon the upper half of the flight chamber indicated upward flight behavior, landing upon the midline denoted horizontal behavior, landing upon the lower half is considered down behavior, and null consisted of a straight drop down into a 5” diameter circle.

Lines obtained from VDRC were 34637 for UAS-hth IR which was crossed to 1151-Gal4 to induce muscle specific effects (Bryantsev et al., 2012). The line, 101052, was used for UAS-Salm IR and was crossed to yw; Mef2-Gal4 which drives muscle specific expression (Dr. Schnorrer, Max Planck Institute of Biochemistry, Martinsried, Germany). UAS-Salm/Cyo;tb was also provided by Dr. Schnorrer and crossed with yw; Mef2-Gal4. UAS-exd and UAS-hth was described earlier and crossed to Actin79B-Gal4 to induce expression in the developing muscle (Bryantsev et al., 2012). Progeny of these crosses were prepared and analyzed for tissue analysis. To visualize founder cells, the animals of rP298-lacZ were used due to its expression of the molecular marker lacZ in
the founder cells (Nose et al. 1998; Ruiz-Gomez et al. 2000). The UAS-aret transgenic line was created by P-element mediated transgenesis by Rainbow Transgenic, Inc. Due to the severity of the phenotype in the aret overexpression studies, a temperature-sensitive inducible driver was used in substitution, Mef2-Gal4; tub-Gal80\textsuperscript{TS}(BDSC stock #7016, McGuire et al. 2003). Activation of aret expression was induced at 48 hours APF (after puparian formation) by switching temperature from 18°C to 29°C. The animals were maintained at the higher temperature until the end of pupal development.

Tissue analysis

Cryosections were made and analyzed as described by Jaramillo et al., 2009. Progeny were prepared by removing the pupal cases from the pupa or taking newly eclosed flies and embedding them into Tissue Tek (OTC) freezing medium (Sakura). Sections were cut at 10 μm in thickness at 18°C using Triangle Biomedical Services Minotome Plus. Sections were fixed in 10% formaldehyde (3.7% v/v) in 1x PBS for 8 minutes. The sections were washed in PBTx (1x PBS, 0.2% v/v Triton-X100). The following antibodies and sera were mixed in 1% BSA (Bovine serum albumin 1% w/v) and incubated at room temperature overnight. Rabbit anti-Aret antibody was provided by Dr. Paul Macdonald at the University of Texas at Austin – ICMB (Kim-Ha et al., 1995), and used at a 1:10 k dilution. The anti-Salm antibody was first described elsewhere (Xie et al., 2007) and used at a dilution of 1:800. The following antibodies and sera were used for identification of nuclear domains: guinea pig anti-Coilin at 1:3k (Joseph Gall, Carnegie Institution, Baltimore, MD), mouse anti-SC35 at 1:800 (Sigma), mouse
anti-Fibrillarin at 1:20 (Abcam), mouse anti-β-galactosidase at 1:1k (Promega), sheep anti-Mbl at 1:5k (Darren Monckton, University of Glasgow, Scotland). Phalloidin was obtained from Molecular Probes, and used at 1:400 dilutions in PBTx. For immunofluorescent detection Alexa conjugated (Molecular Probes) secondary antibodies, Alexa 488 and Alexa 568, were used at 1:400 dilutions and mixed with DAPI (Sigma) at 1 µl/mL. A confocal microscope, Zeiss LSM-780, was used to collect and prepare images. Adobe Photoshop was used to format the digital images that were further assembled in Adobe Illustrator.

Electron microscopy analysis followed an established protocol (O'Donnell et al., 1989). The flies were prepared by removing the head and abdomen, and the thorax was bisected bilaterally for better fixative penetration. The samples were fixed (5% paraformaldehyde in Na₂PO₄ buffer) and processed according to the modifications provided by Dr. Steve Jett at HSC Electron Microscopy Facility at the University of New Mexico-Albuquerque, New Mexico.

Cell culture

*Drosophila* S2 cells were maintained at 25°C in standard Schneider medium (Gibco) containing 10% of fetal bovine serum (HyClone). Transfection assays were performed with TransIT2020 (Mirus Bio) according to manufacturer's instructions with 3 µl per 1 µg of transfected DNA. For normalization, empty vector was used as negative control in co-transfections with candidate minigenes of *sls* and *wupA*. Cells were transfected with *aret* plasmid at a 9:9:2 ratio containing empty plasmid or *aret* plasmid to the minigene plasmid (*sls* and *wupA*) to *pPacPl-Gal4* which equals 1 µg of total DNA. Following
incubation at 25°C, cells were lysed 24 hours after transfection and RNA was extracted. cDNA synthesis followed with RT-PCR analysis to detect changes in splicing events using primers described below.

*Expression analysis*

The flight muscles were extracted in less than one-day old adult flies harboring the genetic knockdown. Muscles were extracted within Tissue Tek (OCT) medium and transferred into lysis buffer supplied by the Qiagen RNeasy Mini extraction kit. All RNA was extracted according to the Qiagen protocol. cDNA was synthesized with Invitrogen Superscript II Reverse Transcriptase using 100 ng of collected RNA, 10 mM DNTP, 5x First Strand buffer, 0.1 mM of DTT, and random hexamer primers (Roche). Diluted cDNA was used as template for subsequent PCR analysis with Pfx Polymerase (Invitrogen) and gene specific primers were used as listed below:

- **sls**: 5’CGCGCAGTATGTGCAAAAT
  5’AAACCGTTCCACGAAAAGTG
- **wupA**: 5’ACACAATCAAATGGCTGATG
  5’GGGGTCATGAAACCCTTCTT
- **Zasp 52**: 5’ATCGCTTCCGACGTTCTGAAG
  5’GTCGCACTAGAGCTTGTTGTTG
- **Zasp 66**: 5’TCCACAAGCAATTCAACTCG
  5’GATACTGGCGCTGATACTGG
- **Act88F**: 5’AGCTCTTCAAAGGCAGCAAC
  5’ATTGTTGTGCGATGGGTTC
PCRs were run at 30-40 cycles. The dilutions of templates were adjusted to loading control (WT-IFM) and amplified equally across all samples using Mhc as a reference gene (Bryantsev et al. 2012b). Final amplification products were visualized on 2% gel.

**Molecular Cloning**

*aret* clone, LD29068, encoding the Aret-PA isoform was obtained from the Drosophila Genomic Resource Center (http://dgrc.cgb.indiana.edu) and sub-cloned into *pUAST-attB* (Bischof et al., 2006) using the Gene Art kit (Invitrogen). The pPacPl-Gal4 construct was created by conventional ligation-based sub-cloning of the Gal4 coding sequence from pAct79B-Gal4 into pPacPl (FBmc0001179, Flybase) at the SpeI sites. To create *sls* and *wupA* minigenes, appropriate genomic fragments were PCR-amplified using the primers listed below and recombined into *pUAST-attB* using the Gene Art kit (Invitrogen).

*sls*: 5'TCGTTAACAGATCTGCGCGCAGTATGTGCAAAATGATCCTCTAGAGGTACAACACCGTTCCACGAAAAGTG;  
*wupA*: 5'TCGTTAACAGATCTGGCCAGTTCTTCTTGAGTTCACCTCTCAGTGAGTTGTGACCCGCTTTG
Letters in bold denote introduced sequences required for product recombination with the vector.

The \textit{s/s} mutant constructs were PCR amplified, with the introduced mutations engineered to generate restriction enzyme sites: NsI for \textit{s/s} region I and SacII for \textit{s/s} region II. The PCR fragments were recombined into pUASTattB using the Gene Art kit. The following primers were used to produce the mutations. The bold sequences are sequences required to recombine the PCR fragment into the vector.

\textbf{sls region I mut:}

5'TAGTTATGCATCCGCTATTAACAAATTATTTCGTGTTTTGTTG

5'AGCGGATGCATAACTAGTGACAAATAATTTTAATGGTTGACA

\textbf{sls region II mut:}

5'TTCTGGACCGCGGAGTTAGGTCTACTATAATGTTGT

5'CCTAACTCCGCGGTCAGAACTTACCTCGATTGTTA

\textit{Bioinformatics}

Exon 10 of the \textit{s/s} gene was used as a query sequence to identify homologous exons in 12 \textit{Drosophila} species performing a BLAST search at the FlyBase resource (FlyBase.org/blast). Sequences adjacent to the identified BLAST hits were aligned against sequences of \textit{D. melanogaster}, and introns flanking \textit{s/s} exon 10 were used in the program AlignX (Vector NTI software suit, Invitrogen). Regions of substantial evolutionary conservation were identified as having 100% nucleotide identity across all of the 12 tested species.
**Statistical Analysis**

All statistical analysis was performed using R Studio. Graphical representation used Microsoft Excel. For the flight data, analysis was performed using success (up behavior) versus failure (all other behavior) using a proportion test. The flies were counted in total with the proportion of upward flight behavior in knockdowns compared to the proportion of upward flight behavior in wild-type flies. Bonferroni correction was utilized for eight different comparisons and the p-value was adjusted to 0.05/8. The analysis resulted in p-values of $1 \times 10^{-25}$, $1 \times 10^{-16}$, $1 \times 10^{-9}$, and $1 \times 10^{-5}$ for *mub*, *aret*, *bl*, and *Spf45*, respectively. The sarcomere length data was measured using Image J software and analyzed using a Wilcoxon-Rank Sum Test and found *aret* knockdown to be significantly different than the wild-type sarcomeres at a p-value of $3.33 \times 10^{-9}$.

**RESULTS**

**Functional test of the aret knockdown**

Identification of potential regulators of alternative splicing required a two-part process. First, a reverse genetic screen was utilized to identify potential regulators of splicing for transcripts in *Drosophila* flight muscles. The genes encoding molecular functions of RNA binding abilities were identified using literature from Schonbauer *et al.* 2012. Transgenic flies harboring a flight muscle specific driver, *Actin88F*, was crossed to UAS-RNAi lines to induce knockdowns of putative regulators. Each knockdown was functionally assessed for flight capability. A loss of flight, categorized as null or down, indicated changes in muscle morphology in these knockdowns. The second condition was identifying changes in alternative splicing using RT-PCR analysis on extracted flight...
muscles harboring the genetic knockdowns. For a regulator to be subjected to further characterization, the animal required a flightless phenotype and alternative splicing changes in the knockdown animals.

The genes for which knockdowns showed significant losses of flight were mub, aret, bl, and spf45, and such animals were tested for changes in alternative splicing. It should be noted that the aret knockdown significantly reduced flight ability and resulted in alternative splicing changes, whereas the other genes did not show a change in alternative splicing (Fig. 1, data not shown).

Validation of aret knockdown

To further confirm the efficiency of the knockdown and its ability to remove Aret expression from the flight muscles, Aret was immunologically detected and counterstained for F-actin and DAPI. The results indicated firstly that Aret accumulates in the flight muscles and not the jump muscles; and secondly that aret knockdown was effective as there was no detection of Aret in the knockdown flight muscles compared to wild-type flight muscles (Fig. 2).
Identification of Aret as a regulator of alternative splicing in flight muscles using RT-PCR analysis

Next, we identified muscle specific genes containing multiply spliced isoforms through flybase.org. Primers were designed to flank each alternatively spliced exon to capture differences in molecular weight for each isoform. A series of candidate genes were utilized to determine the changes in alternative splicing upon removal of aret. These genes were systematically tested and showed clear products for the differential splicing between fiber types, the flight or jump muscle. Figure 3A shows the schematics of the two different alternative splicing patterns detected between the two muscle fibers for each gene tested. The model highlights the different isoform for each muscle type, indirect flight muscles (IFM, flight) or tergal depressor of the trochanter (TDT, jump). cDNA was isolated from
wild-type flight muscle (IFM) and jump muscle (TDT). RT-PCR was used to visualize the change in splicing by using primers designed to detect these splicing changes (Fig. 3B, lanes 1 and 2). The resulting product of the splicing event is presented next to final molecular weight of the different isoforms (Fig. 3C). To gain more perspective on the function of aret, we extracted the muscles from the knockdown of aret, and we examined the splicing patterns within these flight muscles. Upon removal of aret, the transcripts changed from flight muscle to jump muscle specific splicing (Fig. 3B, lane 3), suggesting that without aret the muscle transcripts are converted to jump muscle specific splicing pattern and in turn a role for aret as a regulator of alternative splicing.
Figure 3: Identification of Aret as a regulator of alternative splicing in flight muscles using RT-PCR analysis.

A. Schematics of the different splice patterns within muscle specific genes. Multiply spliced isoforms are indicated with color dashed lines for each muscle type. B. RT-PCR detection for Wild-type flight muscle (IFM), Wild-type jump muscle (TDT), and over knockdown flight muscle (IFM). Note the absence of over leads to jump muscle specific isoforms. C. Schematic of final transcript of exons are indicated for each muscle specific isoform.
**aret knockdown retains flight muscle specific identity**

Next, we wanted to identify whether molecular changes were due to an overall change in muscle identity or due to alterations in the patterns of alternative splicing. cDNA was generated from Wild-type IFM (Fig. 4A, lane 1), Wild-type TDT (Fig. 4A, lane 2), and the isolated flight muscles of the *aret* knockdown (Fig. 4A, lane 3). RT-PCR analysis was performed to detect well-known structural genes for flight and jump muscles. Their expression can be used to identify the muscle type under study. It has been shown Actin88F and TroponinC4 are important for flight muscle function and are expressed exclusively in the flight muscles (Karlik *et al*., 1984; Nongthomba *et al*., 2004). For jump muscles, Actin79B and TroponinC41C, are expressed in the jump muscle and not the flight muscle. (Fyrberg *et al*., 1983; Herranz *et al*., 2004). The analysis determined the products of Actin88F and TpnC4 were retained in the flight muscles of knockdown animals (Fig. 4A, left panel), and the products of Actin79B and TpnC41C were not detected in the flight muscles of the *aret* knockdown (Fig. 4A, right panel). Here, the experiment indicates that the loss of *aret* does not affect expression of the fiber-specific genes, and correct identity is retained in these muscles.

These results indicate that the *aret* knockdown is incapable of flight, and the absence of *aret* leads to jump muscle specific splicing in the flight muscles. From these results, *aret* can be concluded as a regulator of alternative splicing in the flight muscles of the adult fly. Next, we investigated the expression pattern of *aret* to understand when this regulator is required for its splicing function.
are localization in the nuclei of flight muscles and its precursors

To understand aret’s role in muscle development, initial immunofluorescent detection was implemented in the adult fly to detect Aret expression relative to the muscles found within the thorax. The expression of Aret is detected within the flight muscles and is not expressed in the other muscle types within the thorax of the adult fly (Fig. 5A).

Figure 4: aret knockdown retains flight muscle specific identity

A. RT-PCR detection of fiber specific genes from samples of indicated muscle from Wild type IFM (lane 1 in all gels), Wild type TDT (lane 2 in all gels), and aret knockdown IFM (lane 3 in all gels). Actb 5EF and TpnC4 are important genes for flight muscle identity (left panel). For comparison, Act79B and TpnC4IC are important genes for jump muscle formation (right panel). Flight muscle identity is retained in the aret knockdown and changes observed are due to changes in splicing events.

aret localization in the nuclei of flight muscles and its precursors

To understand aret’s role in muscle development, initial immunofluorescent detection was implemented in the adult fly to detect Aret expression relative to the muscles found within the thorax. The expression of Aret is detected within the flight muscles and is not expressed in the other muscle types within the thorax of the adult fly (Fig. 5A).

The temporal expression of the protein was assessed using a reporter gene, rp298-LacZ that selectively expresses Duf in the founder cells within the early developing pupae (Nose et al., 1998; Ruiz-Gomez et al., 2000). At 16 hours APF (after puparium formation), the founder cells begin to establish muscle identity within the future developing muscle, and we wanted to characterize Aret expression at this early time point. Aret expression was not detected in the founder cells of the developing jump muscle at 16 hours APF (Fig. 5B, left panel). However, the expression of Aret within the flight muscles was robustly detected (Fig. 5B, middle panel). This suggests that Aret is solely localized to the developing flight muscles of the developing pupa. The expression of Aret is initiated early during pupal development within a specific nuclear domain. This suggests Aret acts in the nucleus of the flight muscles to perform its regulatory function.
function during alternative splicing of muscle transcripts. Around 24 hours APF, myoblast fusion begins to occur where the fusion competent myoblasts begin fusing with the founder cells to begin forming the muscle fiber. At this time point, Aret expression occurs throughout the nucleus of the founder cells (Fig. 5B, right panel) and this pattern persists into 96 hours APF which might indicate that Aret functions early in development.

Figure 5: Aret localization in the nuclei of the flight muscles and its precursors
A. In 96 h APF pupae, Aret accumulates in nuclei of flight muscles but not jump muscles. Asterisks denote flight muscles, jump muscle is outlined. Scale bar = 50 μm.
B. At 16 h APF, jump muscle founder cells do not accumulate Aret (arrow), whereas the nuclei of flight muscle founder cells (β-gal) accumulate Aret in a nuclear domain (arrowhead). Merged panel highlights founder cell (red) and Aret expression (green) within the nucleus. Aret expression persists in the flight muscle precursors where it diffuses throughout the nucleus in 24 h APF pupa. By this time Aret protein is detected in the nuclei of founder cells that have fused with fusion competent myoblasts (merged). Scale bar = 5.0 μm.
Characterization of Aret expression in flight muscle founder cells

Using the Aret antibody generated by Kim-Ha et al., 1995, we found Aret expression to be locally accumulated in a nuclear domain within the founder cells of flight muscles in the early developing pupae at 16 hours APF (Fig. 5B, middle panel). Previous data have only shown adult expression of Aret within the germ plasm of developing oocytes (Webster et al., 1997). However, given the results of our study that Aret preferentially accumulated within the nuclei of flight muscle founder cells, we assessed whether or not Aret occurred within particular nuclear storage compartments of the founder cells (Fig. 6A-A’). Thus we further characterized Aret expression in the nucleus by searching for co-localization with known nuclear domain markers in 16 hour APF pupa. To accomplish this, a series of experiments were performed to detect nuclear co-localization with markers of common nuclear domains, using antibodies against Coilin (Cajal bodies), SC35 (nuclear speckles), and Fibrillarin (nucleolus). Based on these analyses, we found Aret does not co-localize with any commonly characterized nuclear domains (Fig. 6A-D”) and instead found that the expression of this protein was concentrated in a previously unknown nuclear domain, that we have named the Bruno Body (B-Body). We wanted to determine if the B-body co-localized with Muscleblind which has been implicated in regulation of alternative splicing in muscle genes, such as Troponin T and alpha-actinin (Vicente –Crespo et al., 2008; Vicente et al., 2007). Here we show the B-body co-localizes with Mbl further suggesting a role of Aret as an alternative splicing regulator within flight
muscles (Fig. 6E-E’’). These findings suggest that the B-body may serve as a novel storage unit for regulators of tissue specific alternative splicing.

Identification of the isoform of aret in the flight muscles

Since the aret gene has multiple transcription start sites and is extensively alternatively spliced itself, we further characterized Aret by determining which isoform functions as a regulator of alternative splicing within the flight muscles. Using RT-PCR analysis on wild-type flight muscles, we were able to conclude the most likely flight muscle specific isoform is the RA isoform of Aret (Fig. 7A). Thus, we suggest the RA isoform may be responsible for Aret’s role in alternative splicing whereas the full length isoform may perform translational control of the oskar gene product within developing oocytes (Reveal et al., 2011; Snee et al., 2008; Kim-Ha et al., 1995). It should be noted that there are multiple functions of
Aret, and our study shows different isoforms are potentially responsible for the varied function as well as cellular context.

**aret knockdowns exhibit ultrastructural changes in flight muscle morphology**

Next, we characterized the flight muscle morphology of the transgenic animals lacking aret in the adult fly. The knockdown of aret resulted in aberrant structure of the flight muscles, otherwise known as IFMs (indirect flight muscles). The IFMs are comprised of the dorsal ventral muscles (DVM) and six dorsal longitudinal muscles (DLM) in the adult. The wild-type fly contained robust
muscles with regularly structured and organized muscle fibers in the IFMs (Fig. 8A). The absence of *aret* did not lead to observable changes in gross morphology. The knockdown yielded normally developed IFMs with proper attachments. The muscles were easily distinguishable as flight muscles and contained evenly dispersed nuclei (Fig. 8B). Upon closer inspection, changes resulting from the absence of *aret* were evident at higher magnification. In cross section, the Wild-type DVMs had uniform myofibrils that are the same size and have an even round shape. The myofibrils were evenly spaced with intercellular boundaries (Fig. 8C). In the wild-type DLMs, the myofibrils were neatly arrayed vertically with uniform size and distribution. The H zones are not held in close register between adjacent myofibrils, which is a hallmark of these muscles (Fig. 8D). The knockdown DVMs results in myofibrils that were non-uniform in size and formation. The gaps in intercellular boundaries appear wider, and the most striking feature were the donut shaped myofibrils in the DVMs (Fig. 8E, white arrows). The *aret* knockdown also resulted in wider and narrower myofibrils with synchronous H zones indicating a different striation pattern than in wild-types (Fig. 8F).

The disorganized and aberrant muscle morphology was further characterized using electron microscopy. Compared to controls (Fig. 8G), the DLMs of the knockdowns had wavy Z-lines with broken areas. The lines lost their density and appeared fuzzy. The knockdown showed significantly shorter sarcomeres than in control (*aret* KD: 1.0 µm ± 0.06, control: 3.5 µm ± 0.05) and the M-lines were missing from these structure (Fig. 8H, arrowhead). The electron
micrographs also revealed invasions of cytoplasmic structures within the myofibrils (Fig. 8H, arrow). In comparison, the Wild-type control DVMs contained the hallmark structure of the IFMs. Each thick filament was surrounded by six thin filaments leading to hexagonal shape arrangement in properly formed myofibrils (Fig. 8I, inlet). The electron micrographs further illustrated the significant disorganization of the aret knockdown. Each thick filament was surrounded by a varying number of thin filaments with a loss in the symmetry normally observed in control (Fig. 8J, inlet). The DVMs appeared to be larger than in controls, and there were enigmatic granules within the loosely packed myofibrils (Fig. 8J, arrow). The center of the myofibril in the DVMs contained mitochondria (Fig. 8J, M), which may have served as a central axis upon which fusion could occur between myofibrils. These knockdown muscle fibers seemed to lack strict boundary associations as seen in the Wild-type controls and the resulting effect were larger, fused myofibrils.

The lack of obvious abnormalities seen at the gross morphology level indicates that the inherent characteristic of flight muscle identity was retained. However, closer inspection revealed the proper muscle structure is altered as seen in the loss of the hexagonal arrangement that is a hallmark of IFMs (Figure 8J, inlet). The muscles of the aret knockdown contained a different style of IFM structure and formation, and the altered muscle morphology were due to ultrastructural changes resulting from improper splicing of flight muscle transcripts.
Aret is sufficient in the jump muscles and in tissue culture cells to enforce flight muscle specific splicing.

To comprehend the full effect of aret, we expressed the gene in the jump muscle where it is not normally expressed, and the panel of candidate splice genes was re-implemented to identify changes in splice patterns. RT-PCR was performed on Wild-type IFM (Fig. 9A, lane 1 in all gels), Wild-type TDT (Fig.9A, lane 2 in all gels), and TDT ectopically expressing aret (Fig.9A, lane 3 in all gels). Aret was expressed at 48 hour APF in the developing pupae to bypass the aberrant phenotype seen when aret was expressed in younger pupae (data not shown). The results indicated that the presence of Aret in the TDT leads to...
prominently flight muscle specific splicing with some residual TDT isoforms (Fig. 9A).

To follow the protocol established earlier, the cDNA of the jump muscles were molecularly analyzed for retention of structural identity genes of the jump muscle. RT-PCR was used to detect specific structural genes in Wild-type IFM (Fig. 9B, lane 1 in all gels), Wild-type TDT (Fig. 9B, lane 2 in all gels), and the ectopically expressing aret TDT (Fig. 9B, lane 3 in all gels). The TDT containing aret did not show expression for the flight muscle specific structural genes (Fig. 9B, upper panel). The aret expressing TDT contained proper jump muscle specific structural genes (Fig. 9B, bottom panel). The data indicated that the identity of the jump muscle was retained, and the splicing changes were due to manipulation of alternative splicing as opposed to transformation of muscles.

Next, we tested whether aret could promote flight muscle splicing choices in the naive cellular environment of S2 cells. The aret construct along with candidate minigenes were co-transfected in S2 cells. The minigenes contained portions of the constitutive exons and alternative exons of sls or wupA within a pUAST plasmid. The minigenes were expressed in S2 Drosophila cells to determine if the presence of Aret would lead to flight muscles splicing pattern in muscle specific minigenes, such as sls and wupA. RT-PCR was used to assess the resulting splice events within non-transfected cells, (Fig. 9C, lane 1 in all gels), cells transfected with the minigene constructs (Fig. 9C, lane 2 in all gels), and co-transfection of aret and the minigene constructs (Fig. 9C, lane 3 in all gels). In the absence of Aret, both minigenes were spliced in a jump muscle
specific pattern, indicating that this might be the default splicing pathway for each gene. In the presence of Aret, \textit{sls} transcripts yielded a flight muscle specific pattern (Fig. 9C, left panel). This was also the case in the \textit{wupA} minigene that contained the product of the flight specific patterned isoform (Fig. 9C, right panel). The \textit{wupA} minigene, in the absence of Aret, contained a 550 bp band, which is due to intronic inclusion for the jump muscle/native isoform (Fig. 9C, middle lane). This was a result of an artifact within the minigene construct of \textit{wupA}. Overall, these experiments confirmed that \textit{aret} is powerful enough to induce flight specific splicing and functions effectively in these ectopic environments.
Identification of RNA sequences for Aret-RNA interaction

Aret has been established as an independent regulator of flight muscle alternative splicing. Next, we wanted to identify the regulatory elements within the candidate gene, sls that mediate the effects of Aret function. A schematic of Region I and Region II was shown in Fig. 10A to give the full relation between the exons and introns of the sls gene. Region I and Region II reside in the introns flanking exon 10 (Fig. 10A). We compared sequences of 12 Drosophila species to find regions of interest in the sls gene sequence. The highlighted yellow and
blue areas indicate regions of absolute and significant conservation within the *Drosophila* species. The area outlined in red are potential Aret binding sites, the putative site located at the 5' intronic sequence before exon 10, and one located within the 3' intronic sequence after exon 10. The sequence labeled BP is the conserved branch point required for the basic function of alternative splicing (Fig. 10B). We created constructs with mutations within these sites. Initially, mutation of the branch point (BP) was analyzed to detect if the method was sufficient to determine defects in alternative splicing. Upon mutation of the branch point, the resulting RT-PCR revealed an improper splicing of 5' intron retention in the final transcript (data not shown). Next, three constructs were created with one harboring mutations for region I, another for region II, and the last containing mutations for both regions. These were co-transfected in cells to express the *sls* minigene mutants and the expression plasmid for *aret*. We detected the resulting splice events with RT-PCR. For comparison, the Wild-type *sls* minigene is shown with the predominant product of flight muscle specific splicing (Fig. 10C, panel 1). In Region I, mutation within the first intron yielded split results with a partial conversion to jump muscle specific splicing pattern and residual flight muscle specific splicing in the presence of Aret (Fig. 10C, panel 2). Thus, this mutation was not sufficient to completely block the function of Aret. Next we examined the mutation in Region II, which resulted in jump muscle specific isoform and retention of the upstream intron within the final transcript in the presence of Aret (Fig. 10C, panel 3, red asterisk). Such results suggested the mutated binding site renders an improper splicing event and lack of recognition
for intronic exclusion in the final transcript. To fully characterize Aret and its RNA interaction, a construct with a double mutation was created. The RT-PCR analysis yielded complete transition from flight muscle specific to jump muscle specific splicing even when Aret was present (Fig. 10C, panel 4). These results indicate that individually, the mutation of Reg I and Reg II only attenuate correct Aret-dependent s/l mRNA splicing, while disruption of both regions eliminate flight muscle specific splicing directed by the presence of Aret. The results of our mutation studies may support a model where Aret binds to intronic RNA sequences on either side of a regulated exon to coordinate fiber-specific splicing.
**aret is found within a transcriptional framework of muscle identity**

Previous studies showed that the *Hox* co-factors, *hth* and *exd*, along with the transcription factor, *salm*, encode regulators that are required for proper muscle identity. The *exd* and *hth* work in concert to promote flight muscle specification and are upstream to *salm*. When the *Hox* co-factors are absent in the flight muscles, the muscles lose their unique identity program and take on the morphology and molecular characteristics of the jump muscle. The loss of *salm* results in a transformation of the flight muscle to the tubular leg muscle type. These identity changes lead to loss of muscle function and lethality of the fly (Bryantsev et al., 2011; Schonbauer et al., 2011). Preliminary work by Schonbauer et al., 2011, has suggested *aret* may fall within this regulatory framework due to its decreased expression when *salm* is downregulated. *aret* may function in this framework by maintaining the flight muscle type within the adult fly.

By genetically manipulating potential upstream factors, the location of *aret* in this transcriptional framework can be determined. Wild-type flies exhibit *aret* expression in the flight muscles of the adult fly thorax (Fig. 11A-A’). In the *hth* knockdown and *salm* knockdown, there was a reduction in *aret* expression within the transformed flight muscles as detected by immunofluorescence (Fig. 11B-C’). The jump muscles within the thorax lack the expression of *aret* (Fig. 11D-D’). To further determine *aret’s* position in this transcriptional background, we carried out the converse experiment of ectopic expression of *hth* and *salm* within the jump muscles. The jump muscle fibers expressing *hth* and *salm* resulted in muscle
transformation into the fibrillar muscle type and an increase in Aret expression (Fig. 11E-F').

Figure 11: aret is found within a transcriptional framework of muscle identity

aret is downstream to flight muscle identity markers, exd/hth and salm. A-A'. Wild-type flight muscles are used in comparison to knockdowns of hth (1131; Fis-lacZ x UAS-hth IR) and salm (yw; Meg2-Gal4 x UAS-salm IR) with transformed flight muscles. Aret protein (green) accumulation was detected by immunofluorescence within the Wild-type flight muscles. Muscle morphology was assessed using phalloidin to highlight actin filaments (red). Note reduced Aret expression in the transformed muscle fibers of the hth knockdown (B-B') and salm knockdown (C-C'). Scale bar = 20 μm. D-D'. Wild-type jump muscles are used to further determine aret in the transcriptional framework. Aret (green) is not expressed within the Wild-type jump muscles fibers using phalloidin (red). E-F'. When hth (79B-Gal4 x UAS-exd/hth) and salm (yw; Meg2-Gal4 x UAS-salm/Cyo;th) are expressed in the jump muscle, Aret is detected by immunofluorescence. Note the transformed fibrillar-type fibers (red) of the jump muscles accumulate Aret in the nuclei. Scale bar = 20 μm.

Schematic of aret in a transcriptional framework

Based on the data provided here and elsewhere, the following model is proposed. hth and exd can work with salm to establish flight muscle specification. Hth and Exd can work directly on the enhancer of the Actin88F gene, which results in flight muscle identity (Bryantsev et al., 2012). Salm works as a bridge between flight muscle identity and flight specific alternative splicing by working with aret. This process allows salm to further maintain the identity by interacting
with *aret* which regulates proper alternative splicing among flight specific gene products (Fig. 12A). These results place *aret* in the known genetic network controlling flight muscle identity, thus providing an important link between transcriptional controls and splicing control during muscle fiber specification.

**DISCUSSION**

The mechanism of determining muscle fiber types by alternative splicing is an uncharacterized process in the developing fly. Our findings demonstrate the profound effect of Aret upon muscle specific alternative splicing. The protein works autonomously to promote flight muscle specific alternative splicing. Our studies indicate Aret’s removal results in flightless behavior due to the dramatic changes within the molecular signature and the ultrastructural formation of the resulting muscle. Previous work has shown the importance of correct splice events in mammals. Incorrect splicing can lead to muscular dystrophies due to the occurrence of improper fiber switches (Berger *et al.*, 2011) and result in
cardiomyopathies due to the presence of fetal isoforms in the developing heart (Ladd et al., 2005a). Alternative splicing is an important mechanism for regulating developmental switches during muscle development. Our studies further identify the impact Aret has upon the regulation of specific muscle fiber types, and implicates Aret as an influential regulator of alternative splicing in the adult flight muscles of Drosophila.

In our research, we have discovered a specific nuclear domain for Aret within the nuclei of the flight muscle founder cells. This domain is unique due to lack of co-localization with any other known nuclear domain, such as Cajal bodies and nuclear speckles. However, it should be noted the B-body co-localizes with a previously studied protein, Mbl, which has been implicated as a regulator of alternative splicing in muscle genes and in the genetic disease, Myotonic Dystrophy (Wang et al., 2012). It would be interesting to further elucidate the complex protein network of alternative splicing regulation starting with the interaction of Aret and Mbl.

Furthermore, we have identified new regulatory elements within a model muscle gene to determine binding sites for Aret’s function in muscle specific alternative splicing. Through our bioinformatics analysis, we were able to establish specific regions in the s/s introns where Aret functions to promote flight muscle splice events. Further investigations would detect additional regulatory elements in the introns of other muscle specific genes to identify other regulatory proteins and RNA sequence elements involved in the vast network of muscle development.
In a recent study, Schonbauer et al. 2011, has characterized a regulatory network of muscle identity by indicating Salm is an important regulator in the flight muscles of the developing fly. Their findings suggest the absence of Salm results in another fiber switch from fibrillar to tubular muscle type (2011). Another study has expanded this regulatory network by including certain hox gene co-factors, exd and hth. These genes are important for fiber identity of the flight muscles within the adult fly, where their absence results in a switch in identity from fibrillar type to tubular type (Bryantsev et al., 2012). The resulting transcriptional network from these two studies has shown Exd and Hth work upstream to Salm, and these factors work in concert to establish correct muscle identity. Our studies further characterize this transcriptional framework by establishing that Aret helps maintain the flight muscle identity through promotion of flight specific alternatively spliced genes.

Since regulation of alternative splicing has been studied in vertebrate somatic and cardiac muscles, our findings may relate to processes of muscle diversification through differential splicing of specific muscle genes in vertebrates. In mice, there is coordinated expression of CELF1 and CELF2 with Mbnl in heart development. These proteins promote proper cardiac development by regulating splicing during developmental switches; however, CELF1/2 overexpression and Mbnl knockouts result in mis-splicing in many cardiac gene products. The consequence is a shift toward embryonic/early postnatal isoforms (Kalsotra et al., 2008). Further studies indicate the need for balanced expression of CELF. When this regulator is improperly maintained, there are incorrect splice events in many
muscle genes, which results in Myotonic Dystrophy (Dhaenens et al., 2011; Charlet et al., 2002; 1988; Philips et al., 1998; Savkur et al., 2001). The relative high prevalence of Myotonic Dystrophy and congenital heart diseases indicate a need to understand how factors, such as Aret, perform their regulatory functions. Through this conserved process, Drosophila can improve the understanding of the critical pathways that mediate splicing by identifying causes of splicing defects. Most critically, Aret can identify novel regulatory pathways in Drosophila that can be translated to vertebrates.
REFERENCES CITED


