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

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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

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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.

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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. RRMs 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^{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 Na_2PO_4 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'ACACAAATCAAAATGGCTGATG

5'GGGGTCATGAAACCCTTCTT

Zasp 52: 5'ATCGCTTCCGACGTTCTGAAG

5'GTCGCAGTAGAGCTTGTTGTTG

Zasp 66: 5'TCCACAAGCAATTCAACTCG

5'GATACTGGCGCTGATACTGG

Act88F 5'AGCTCTTCAAAGGCAGCAAC

5'ATTGTTGTGCGATGGGTTC

TpnC4 5'TGGCAGCTCGCTTTATTG

 5'GTGTTGCAACTGTCAGGTATCC

Act79B 5'TGTCTCCAGCGTAAGACATCC

5'TTCCGGTCTTTTCTCGTCTC

TpnC41C 5'CGCCTTTACGACAAAGAAGG

5'CATGTCCAGGTCGTCATTTG

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'**TCGTTAACAGATCTGC**CGCGCAGTATGTGCAAAAT

5'**GATCCTCTAGAGGTAC**AAACCGTTCCACGAAAAGTG;

wupA: 5'**TCGTTAACAGATCTGC**CCAGTTCCTTGAGTTCACCTCTC

5'**GATCCTCTAGAGGTAC**GTCTACGTTCAGCCGCTTTG

Letters in bold denote introduced sequences required for product recombination with the vector.

The *sls* mutant constructs were PCR amplified, with the introduced mutations engineered to generate restriction enzyme sites: NsiI for *sls* region I and SacII for *sls* 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.

sls region I mut:

5'TAGTTATGCATCCGC**TATTAACAAATTATTTCGTGTTTTGTTG**

5'AGCGGATGCATAACT**AGTGACAAATAATTTTAATGGTTGACA**

sls region II mut:

5'TTCTGGACCGCGGAG**TTAGGTCTACTATAAATGTTGT**

5'CCTAACTCCGCGGTC**CAGAACTTACCTTCGATTGTTA**

Bioinformatics

Exon 10 of the *sls* gene was used as a query sequence to identify homologous exons in 12 *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 *D. melanogaster,* and introns flanking *sls* 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 .05/8. The analysis resulted in p-values of 1×10^{-25}, 1×10^{-16}, 1×10^{-9}, and 1×10^{-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 3.33×10^{-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).
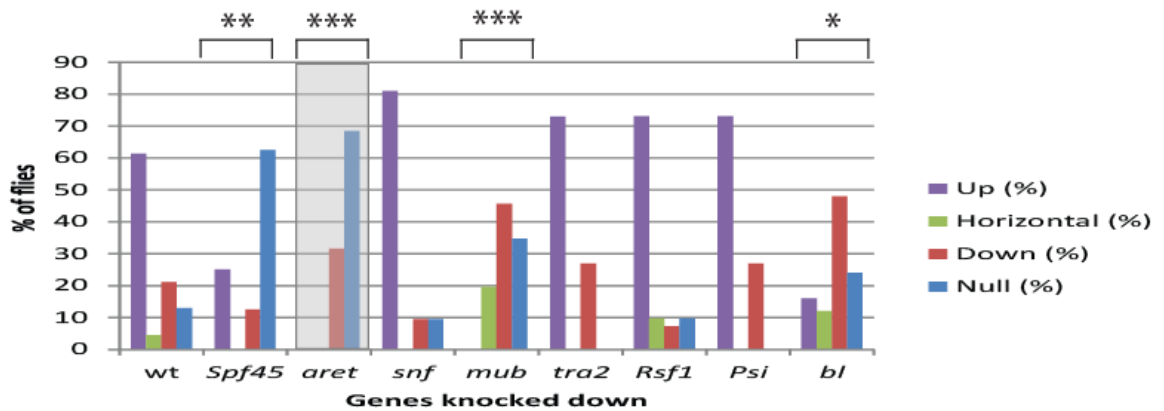
Figure 1: Functional test of aret knockdown
A. Histogram of the functional test performed on knockdowns of putative regulators of alternative splicing. Flight ability was assessed in flies crossed with Wild-type flies and knockdowns of *aret, Spf45, snf, mub, tra2, rsf1, Psi,* and *bl*. Each knock down was assessed for flight ability: null, down, horizontal, or up. X-axis is genes knocked down, y-axis is percentage of flies that were normalized to 100% per phenotypic category. * = p-value < 1e-5, ** = p-value < 1e-9, *** = p-value < 1e-16.

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).

A
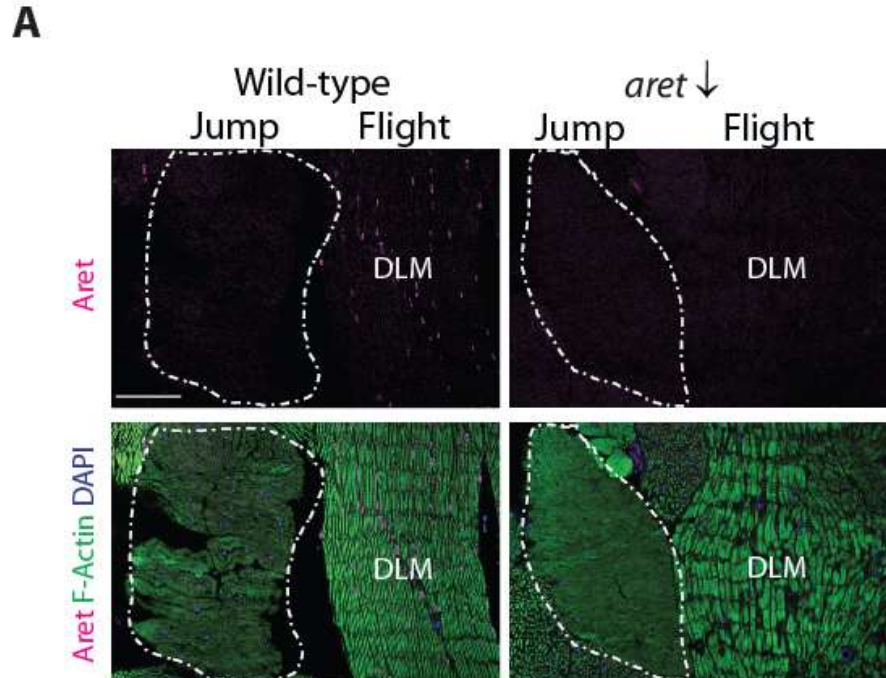
Figure 2: Verification of the *aret* knockdown

A: Immunological detection of Aret expression (magenta) in control (Wild-type) and *aret* knockdown flight muscles (DLM). Jump muscle (TDT) is outlined in white dashes. Muscle morphology is detected by using F-actin (green) and nuclei (blue). Note that the knockdown muscles do not accumulate Aret. Scale bar =50 μm

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.
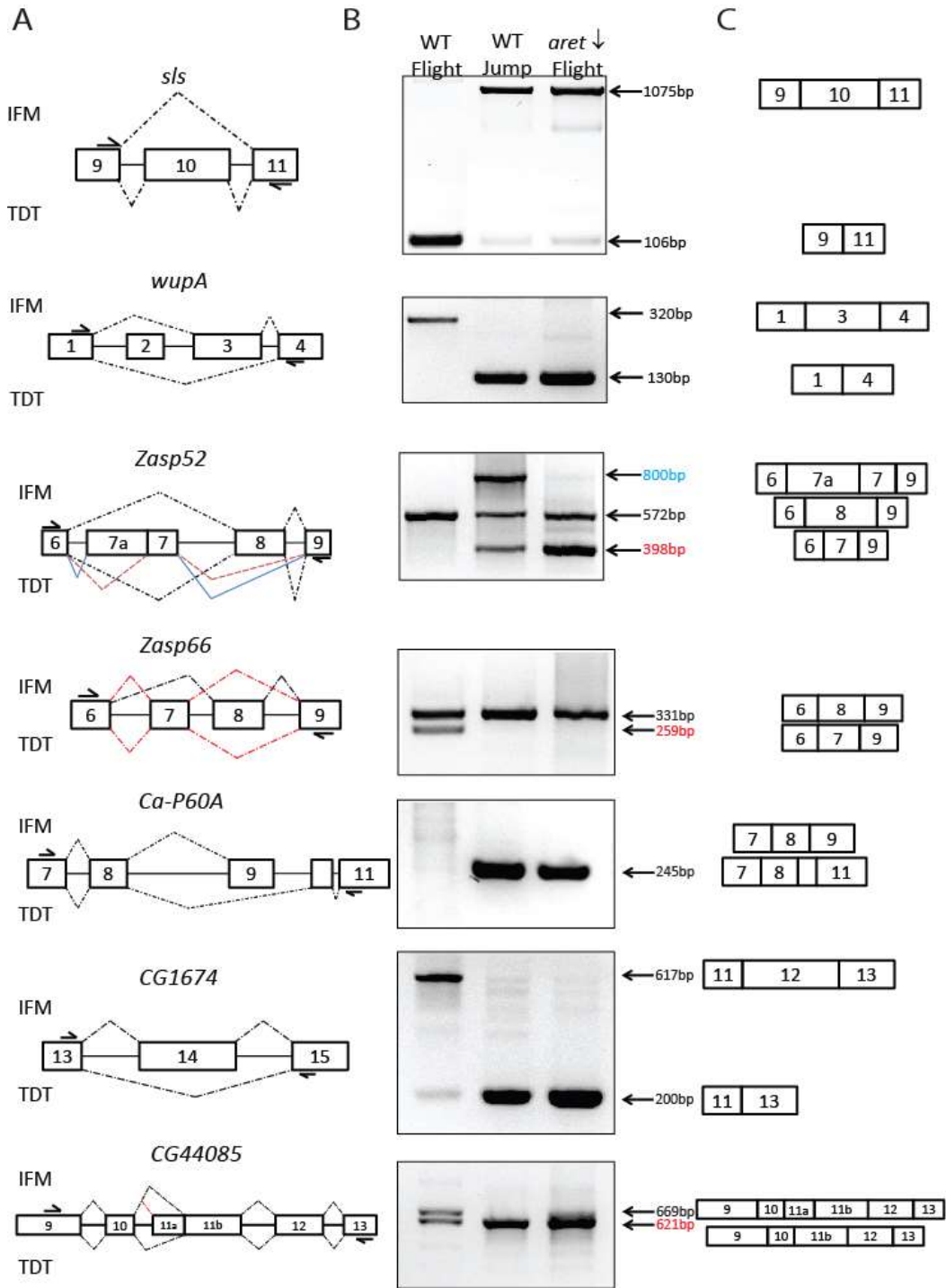
Figure 3: Identification of Aret as a regulator of alternative splicing in flight muscles using RT-PCR analysis

A. Schematics of the difference 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 *aret* knockdown flight muscle (IFM). Note the absence of *aret* 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.

A
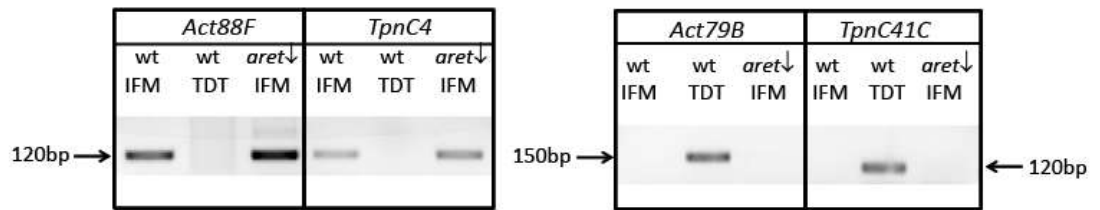
Figure 4: *aret* knockdown retains flight muscle specific identity
A. RT-PCR detection of fiber specific genes from samples of indicated muscle from Wild-type IFM (lane 1 in all gels), Wild-type TDT (lane 2 in all gels), and *aret* knockdown IFM (lane 3 in all gels). *Actin 88F* and *TpnC4* are important genes for flight muscle identity (left panel). For comparison, *Actin79B* and *TpnC41C* 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

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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.
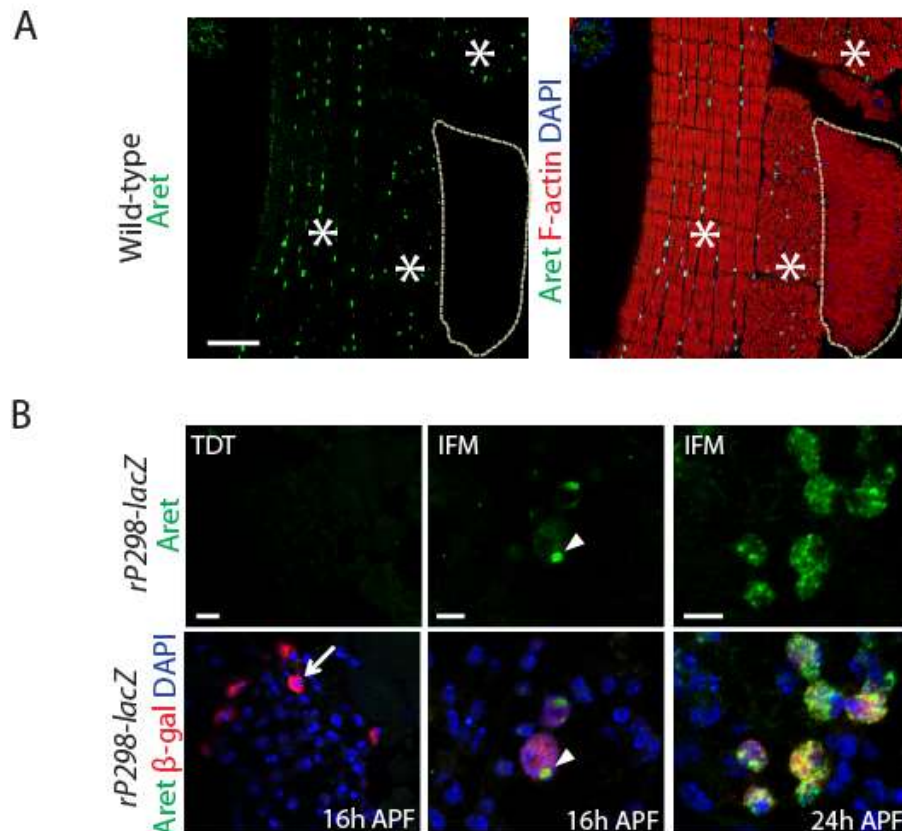
Figure 5: Aret localization in the nuclei of the flight muscles and its precursors
A: In 96 h APF pupae, Aret accumulates in nuclei of flight muscles but not jump
muscles. Asterisks denote flight muscles, jump muscle is outlined. Scale bar = 50 μm.
B: At 16 h APF, jump muscle founder cells do not accumulate 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 is
diffuses throughout the nucleus in 24 hr 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.
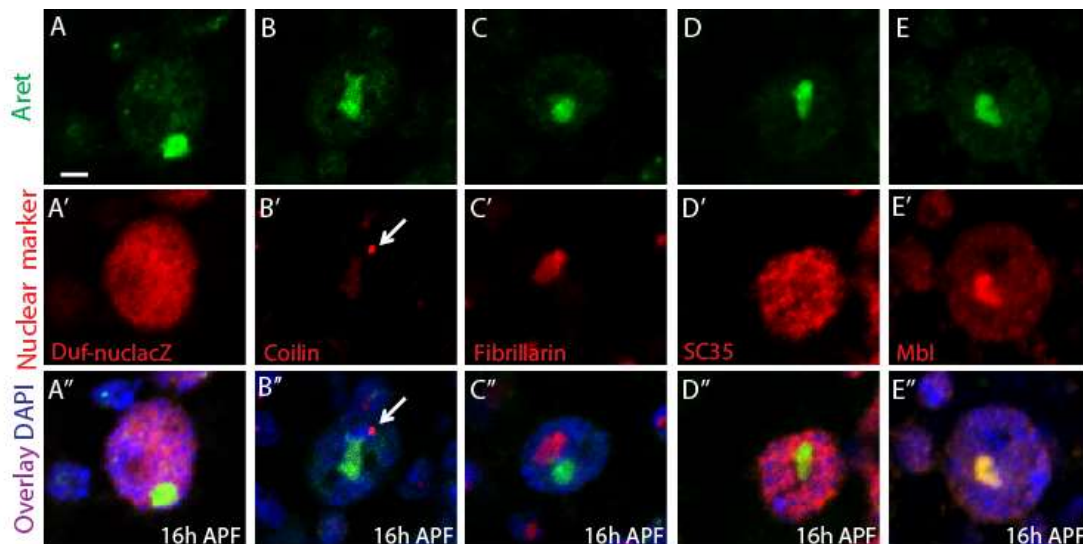
Figure 6: Characterization of Aret in flight muscle founder cells
Aret is locally isolated to an unknown nuclear domain in early pupal development and diffuses throughout the nucleus later in development. A-A''. Aret (green) accumulates in the nuclei of 16 hr APF pupa using the *rp298-nucLacZ* transgenic line as visualized with immunofluorescence. In the first panel *Duf* expression (red) marks the flight muscle founder cells which coincides with large nuclei (DAPI). Note that Coilin (B-B'', arrow), Fibrillarin (C-C''), and SC35 (D-D''), marks cajal bodies, nucleolus, and nuclear speckles, respectively. Aret does not co-localize in any of the nuclear compartments but resides in an unknown domain, B-Body, Bruno-Body. However, the B-body co-localizes with the protein Mbl (E-E''). Scale bar = 2.0 μm.

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.
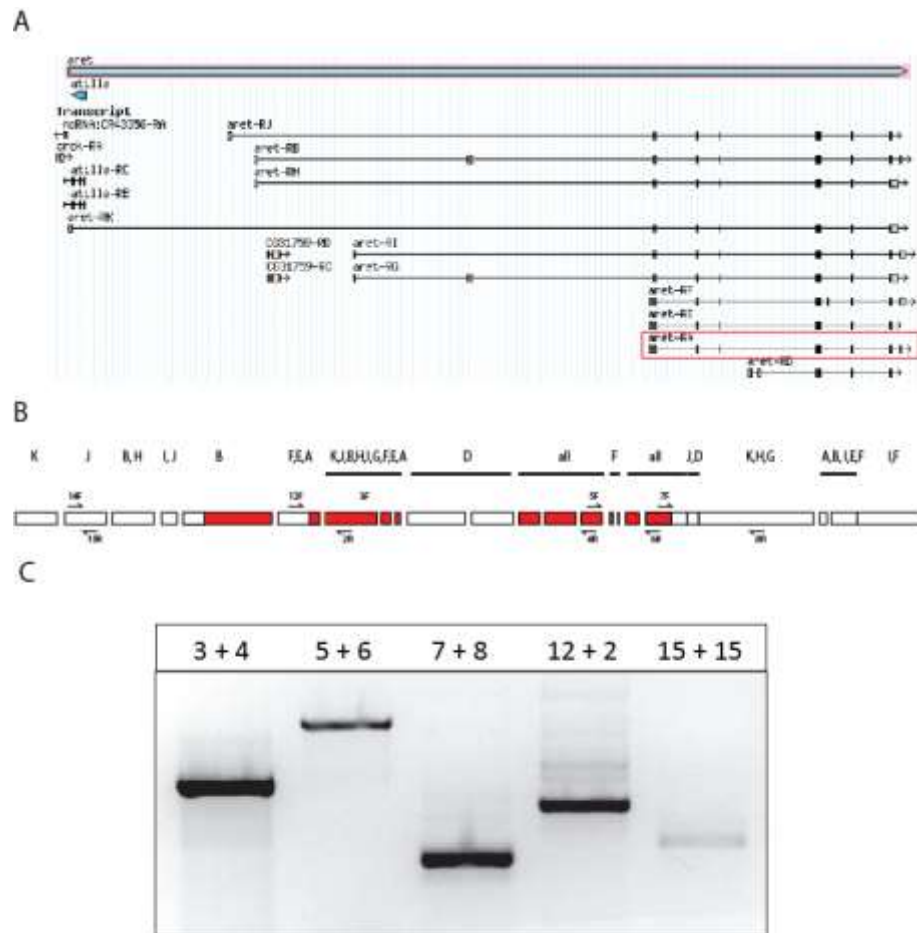
Figure 7: Identifying the isoform of *aret* in flight muscles
A. Genomic structure of the *aret* gene (flybase.org). Notice the aret gene is alternatively spliced. B. Genomic model indicating primers and isoforms tested. Red indicated translated exons and letter and lines above genes indicate isoforms tested against the exonic regions in the *aret* gene. RA is the isoform found in the adult flight muscles. C. RT-PCR analysis of wild-type flight muscle samples against primers listed above (B). Numbers indicate primer pairs used in amplification.

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.
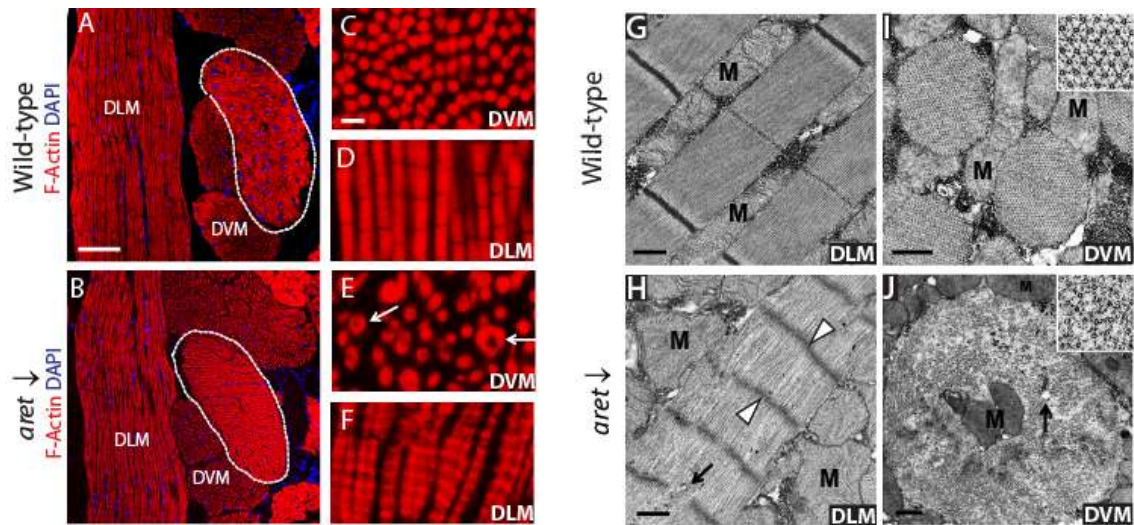
Figure 8: *aret* knockdown exhibit ultra-structural changes in morphology of flight muscles
The morphology of the *aret* knockdown was assessed using immunofluorescence to indicate the absence of this regulator leads to improper splice events in the flight muscles of the adult fly. A. Adult myofibrils are detected with fluorescent phalloidin (red) to highlight fiber specific features of the flight and jump muscles (outlined in white dashes). Note the morphological features of Wild-type controls in comparison to the muscle structure of the aret knockdown, *Actin88F-Gal4* x *UAS-aret* IR (B). At gross morphology, no obvious changes have occurred. Scale bar =50 µm. The morphology was examined at the individual myofibril level with phalloidin (red) highlighting the Wild-type muscles. C -D. The muscles have organized structural formations in the controls. E-F. The *aret* knockdown exhibits ultra-structural changes in muscle structure and organization. Note the donut formation of myofibrils in the knockdown DVMs (E, white arrows). Scale bar = 5.0 µm. G-I. Shape and organization of myofibrils were visualized with electron microscopy of Wild-type controls and the *aret* knockdown. Notice the hallmark formation of the IFM with thin filaments surrounded by six bundles of thick filaments leading to the hexagonal shape of a properly formed myofibril (I, inlet). H-J. The *aret* knockdown has shorter sacromeres in the DLM (white arrowheads) and lack the hexagonal shape (H, inlet). There are larger fibrils in the DVM (J). Note the mitochondria are located within the donut formations seen in the DVMs. H. Arrow= cellular structures. J. arrow = granules. M = mitochondria. Scale bar = 0.5 µm.

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, *sls* transcripts yielded a flight muscle specific

pattern (Fig. 9C, left panel). This was also the case in the *wupA* minigene that

contained the product of the flight specific patterned isoform (Fig. 9C, right

panel). The *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

wupA. Overall, these experiments confirmed that *aret* is powerful enough to

induce flight specific splicing and functions effectively in these ectopic

environments.
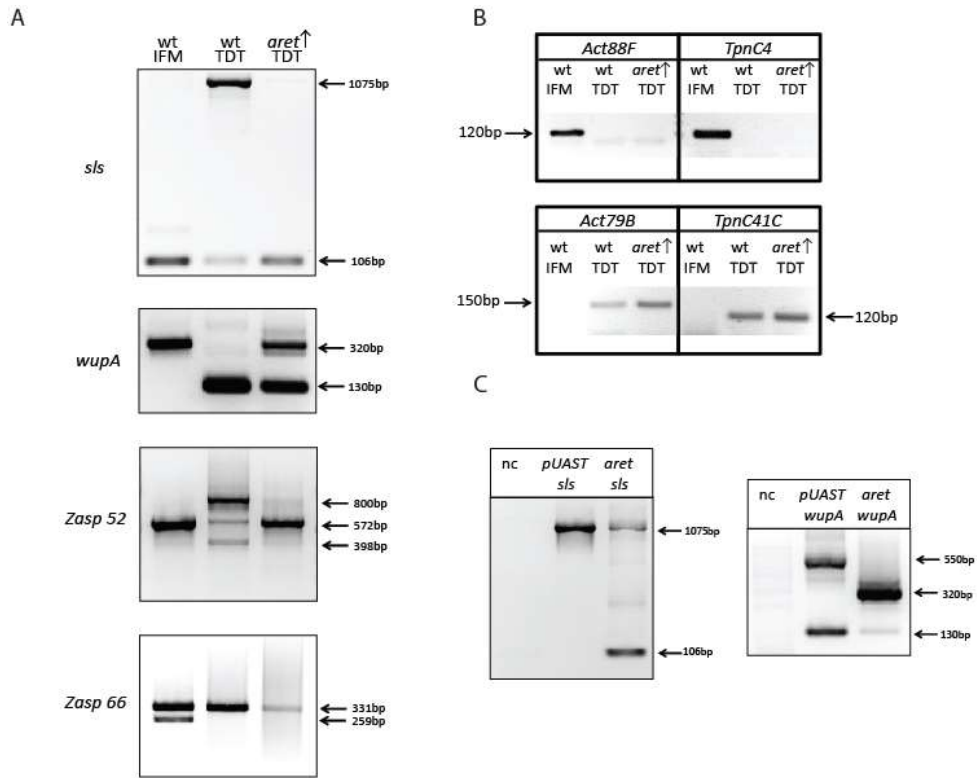
Figure 9: Aret is sufficient in the jump muscles and tissue culture to enforce flight muscle specific splicing.
Aret can autonomously promote flight muscle patterns of splicing in the jump muscle and S2 cells. A. *aret* was ectopically expressed in the TDT, and the patterns of fiber specific splicing for candidate splice gene products were analyzed using RT-PCR. Wild-type flight muscle (IFM, lane 1 in all gels), Wild-type jump muscle (TDT, lane 2), are provided in comparison with the ectopically expressing *aret* jump muscle (TDT, lane 3). The presence of Aret in the jump muscle shifts the products towards the flight muscle isoform and suppresses the appearance of the jump muscle splice products. B. Ectopic expression of *aret* in the jump muscles does not alter the muscle identity of the muscle genes that are independent of alternative splicing. RT-PCR detection of fiber specific genes from samples of indicated muscle from Wild-type IFM (lane 1), Wild-type TDT (lane 2), and ectopically expressing *aret* TDT (lane 3). C. Aret promotes flight muscle splicing choices in the naive cellular environment of S2 cells. Gene specific RT-PCR was utilized to identify Aret's role of inducing flight specific splicing patterns in the *sls* and *wupA* mini-genes. For comparison, RT-PCR was performed no- transfected cells (lane 1 in all gels), and S2 cells with transfected mini-gene constructs (lane 2). The S2 cells were co-transfected with the mini-gene constructs and *aret* (lane 3). Note that for both *sls* (left panel) and *wupA* (right panel) the mini gene is spliced in a manner similar to the endogenous gene in the jump muscles, but the co-expression of *aret* results in a strong shift towards flight muscle splicing.

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 *sls* 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.
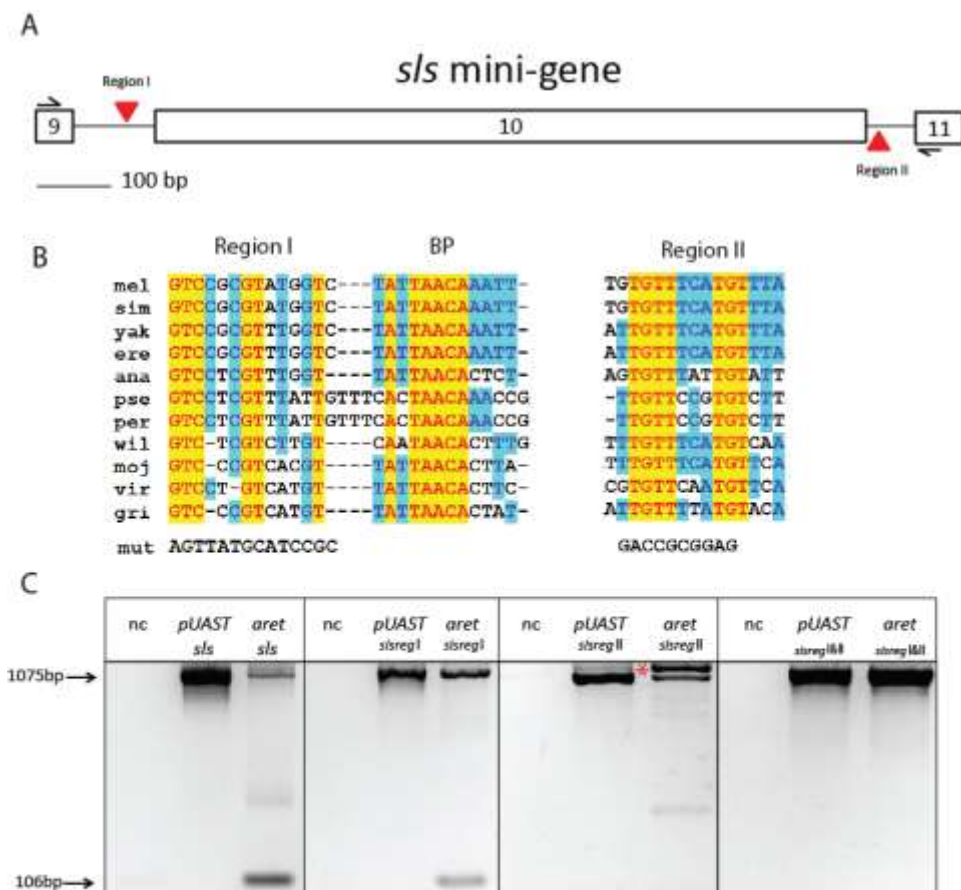
Figure 10: Identification of regions for Aret-RNA interaction
Novel regions of Aret interaction was identified in the *sls* gene product through mutagenesis of evolutionarily conserved sites. A. Schematic of the *sls* mini-gene with indicated red arrows for region I and region II in relation to the exons and introns of the gene. The red arrows indicate potential binding sites of Aret. Scale bar = 100 bp B. Sequence of the *sls* mini-gene showing areas of conservation termed region I and region II. Yellow and blue highlights indicate significant nucleotide conservation in 12 Drosophila species. Red highlighted areas are putative regulatory elements for Aret binding in the first intron (left). BP indicates sequence conservation of the branch point necessary for the basal function of alternative splicing. C. RT-PCR detection of the splicing events resulting from Wild-type *sls*, mutation in region I, mutation in region II, and double mutation of region I and region II. Note that both mutations are sufficient and necessary to block

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').
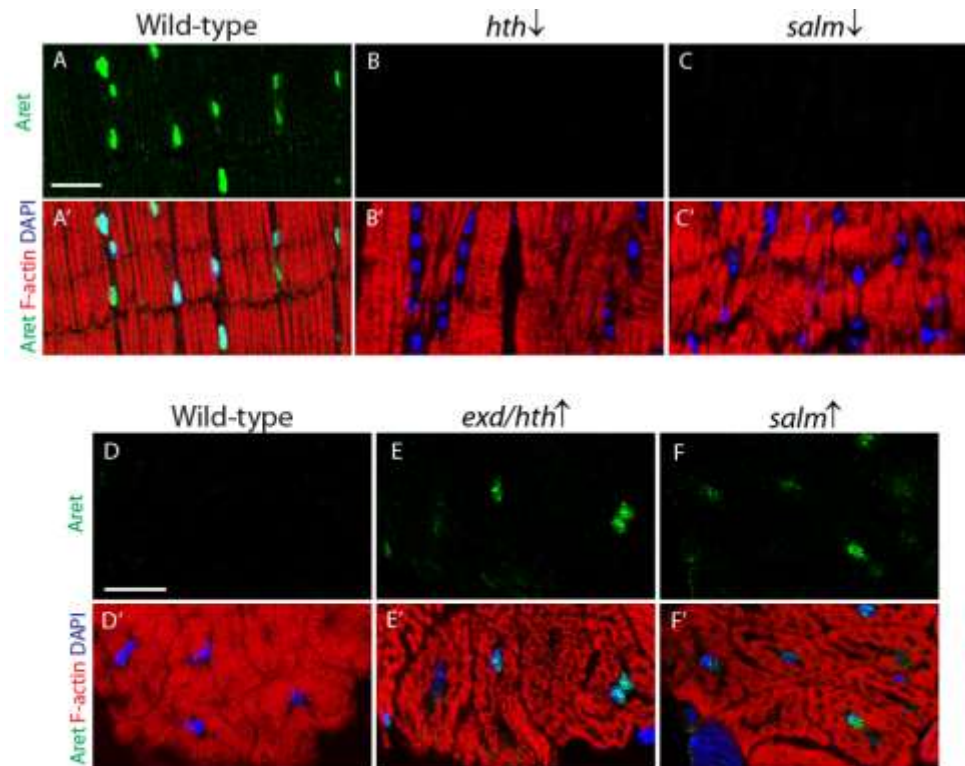
Figure 11: *aret* is found within a transcriptional framework of muscle identity
aret is downstream to flight muscle identity markers, *exd/hth* and *salm*. A-A'. Wild-type flight muscles are used in comparison to knockdowns of *hth (1151; Fln-lacZ x UAS-hth IR)* and *salm (yw; Mef2-Gal4 x UAS-salm IR)* 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; Mef2-Gal4 x UAS-Salm/Cyo;tb)* 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.

A
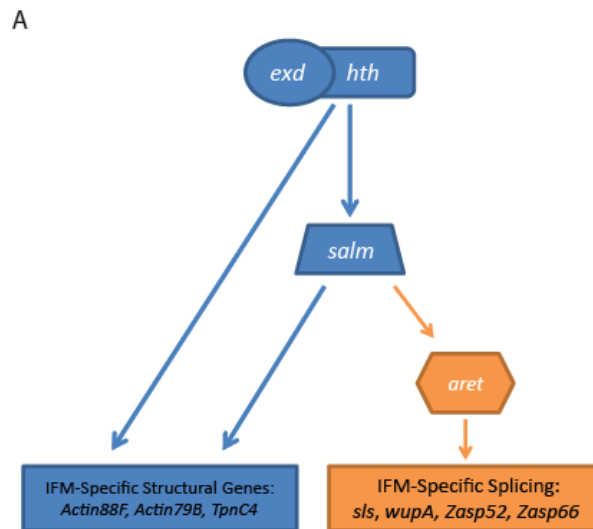
Figure 12: *aret* is downstream to identity genes for flight muscles and funtions in maintenance of flight muscle specific alternative splicing

A. Model depicting identity genes and *aret* in a transcriptional framework. The identity genes, *exd/hth* and *salm* encode proteins that regulate flight muscle structural genes while Aret regulates flight specific alternative spliced muscle genes.

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 *sls* 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

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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.

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