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The gene blistered selectively controls muscle type differentiation in Drosophila melanogaster

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THE GENE *BLISTERED* SELECTIVELY CONTROLS

MUSCLE TYPE DIFFERENTIATION IN *DROSOPHILA MELANOGASTER*

By

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B.S., Biology, University of New Mexico, 2011

THESIS

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ABSTRACT

The muscle fiber is a structural unit of skeletal muscle in many organisms. Muscle fiber composition has been implicated in maintaining muscle longevity and controlling systemic energy homeostasis, however not much is known about how different types of muscle fibers are specified. The fruit fly *Drosophila melanogaster* provides a useful model to study specification and differentiation of different types of muscle fiber. In this study, the functional properties of the transcription factor *blistered* (*bs*) were explored. The gene *bs* is evolutionarily conserved and has a mammalian homologue, Serum Response Factor (SRF). When *bs* is genetically down-regulated in all muscles, only a subset of muscles, called Indirect Flight Muscles, is affected and shows immature and disorganized myofibrils. The development of other muscles proceeds normally. To understand the role of *bs* in the selectivity towards Indirect Flight Muscles, I focus on identification of its targets, potential cofactors and the mechanism that governs fiber differentiation in skeletal muscle. Results suggest *bs* works in conjunction with pioneer factors, *exd* and *hth*, to regulate expression of flight muscle specific *Act88F*, thus providing a mechanism for flight muscle fiber specification.

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ABSTRACT

The muscle fiber is a structural unit of skeletal muscle in many organisms. Muscle fiber composition has been implicated in maintaining muscle longevity and controlling systemic energy homeostasis, however not much is known about how different types of muscle fibers are specified. The fruit fly *Drosophila melanogaster* provides a useful model to study specification and differentiation of different types of muscle fiber. In this study, the functional properties of the transcription factor *blistered* (*bs*) were explored. The gene *bs* is evolutionarily conserved and has a mammalian homologue, Serum Response Factor (SRF). When *bs* is genetically down-regulated in all muscles, only a subset of muscles, called Indirect Flight Muscles, is affected and shows immature and disorganized myofibrils. The development of other muscles proceeds normally. To understand the role of *bs* in the selectivity towards Indirect Flight Muscles, I focus on identification of its targets, potential cofactors and the mechanism that governs fiber differentiation in skeletal muscle. Results suggest *bs* works in conjunction with pioneer factors, *exd* and *hth*, to regulate expression of flight muscle specific *Act88F*, thus providing a mechanism for flight muscle fiber specification.

Keywords: *Drosophila*, *bs*/SRF, muscle differentiation, muscle fiber type

INTRODUCTION

Skeletal muscles of vertebrates are formed through a process known as myogenesis. Myogenesis is a complex process that involves a tightly regulated sequence of events that eventually lead to the formation of a multinucleated muscle fiber. The resulting individual skeletal muscle fibers formed throughout this process are heterogeneous and can be distinguished based on morphology, physiology, and molecular makeup (Schiaffino and Reggiani, 2011; Scott et. al., 2001). Typically, vertebrate muscle consists of two main classes of fibers that are identified as either Type 1 or Type 2. Type 2 is further divided into several subclasses (type 2A, 2B, 2X) (Schiaffino and Reggiani, 2011). Type 1 fibers are abundant in slow-twitch muscles and Type 2 fibers predominantly appear in fast-twitch muscles. The morphological differences among fiber types define the ability of the muscle to adapt to various forms of physical stress, allow for a specific level of endurance, and even have the potential to signify susceptibility to certain chronic myopathies (Zierath and Hawley, 2004). Recent research has also found fiber type composition plays a role in mediating systemic metabolism (Baskin et al., 2015).

Furthermore, muscle fibers also exhibit differences in gene expression that help distinguish fiber types at the molecular level (Spangenburg and Booth, 2003). For example differential expression of genes such as Myosin Heavy Chain, across the different fiber types is currently used as a marker for fiber type diversity (Schiaffino and Reggiani, 2011; Spangenburg and Booth, 2003). Although early stages of myogenesis have been extensively studied, very little is

known about how different types of skeletal muscles are specified and differentiated.

Similarly, *Drosophila* has two major fiber types, fibrillar and tubular. Fibrillar muscles make up the indirect flight muscles (flight muscles) of the adult thorax and function to provide the power necessary for flight; tubular muscles, such as the jump muscles and leg muscles are responsible for walking and the initiation of flight (Bernstein et al. 1993). Expression of fiber-specific genes further validates the morphological differences between both types of fibers in *Drosophila*. For example, tubular muscles express the muscle specific actin gene, *Act79B*, whereas the fibrillar muscles almost exclusively express *Act88F* (Fyrberg et al., 1983; Karlik et al., 1984). Other differentially expressed genes that have not been identified to play a role in the formation of different fiber types may offer insight into possible mechanisms. The morphological and molecular diversity shared among fiber types, along with the conservation of the myogenic framework, make *Drosophila* a valid platform to further investigate questions regarding fiber specification and differentiation of skeletal muscle.

In *Drosophila*, the general steps of somatic muscle development are similar to those seen in vertebrate myogenesis (Bate, 1993). A majority of the muscle in *Drosophila* is composed of somatic muscle that is derived from the mesoderm. Somatic muscles in adult flies arise from a population of adult muscle progenitor cells that proliferate and become myoblasts. These myoblasts receive stimulatory cues to become either fusion-competent myoblasts or founder cells. The founder cells begin to incorporate nuclei of surrounding

fusion-competent myoblasts and later become a multinucleated muscle fiber (Taylor, 2003; Bate, 1993).

Individual muscle fibers that are formed through the process of myogenesis obtain their identity based upon differential patterns of gene expression. For example, *homeotic selector* (*Hox*) genes have been shown to influence fiber-specific gene expression and ultimately impact muscle patterning in *Drosophila* (Roy and VijayRaghavan, 1997). The *Hox* cofactors, Exd and Hth, have been characterized as muscle identity genes and are important for helping to understand the link between muscle development and identity. Specifically, the genes *exd* and *hth*, are responsible for switching fiber identity between two different fiber types in the adult thorax and are direct transcriptional regulators of the flight muscle specific gene *Actin88F* (Bryantsev et al., 2012). Importantly, vertebrate orthologs of *exd* and *hth*, called Pbx and Meis, have also been found to play a role in diversification of vertebrate muscle fibers (Maves et al., 2007). It is not known, however, if proteins Exd and Hth act alone or rely on the presence of additional factors. Further investigation is needed to explain how fate-determining factors carry out muscle type specification mechanistically.

Recent work has identified the transcription factor Serum Response Factor (SRF) as playing a role in skeletal muscle differentiation. SRF is a member of the evolutionarily conserved MADS-domain-containing family of regulators (Shore and Sharrocks, 1995) and has a *Drosophila* homolog encoded by the *blistered* (*bs*) gene (Montagne et al., 1996). Previous studies have identified SRF binding sites, also known as serum response elements, which are

often located in the promoters of muscle genes (Miano, 2003). Functional SRF binding sites contain a conserved DNA sequence [CC(A/T)6GG], known as the CArG box. The ability of SRF to bind the CArG box is determined by a number of factors, including presence of the complete CArG consensus sequence and association with cofactors. Target enhancers bound by SRF, and thus regulated by the transcription factor, include those involved in cell growth, cardiac differentiation, the actin cytoskeleton, and skeletal muscle differentiation (Miano et al., 2007).

The abundance of SRF in vertebrate muscle is associated with the regulation of several tissue-specific target genes. One gene that tends to be expressed in response to the presence of SRF is actin. In vertebrates, SRF has been classified as a master regulator of the actin cytoskeleton and has been shown to be necessary for cellular growth (Miano et al., 2007). The promoter region of *Actin403* from the arthropod *Artemia franciscana* contains the CArG binding site and it is known to bind SRF at this site (Casero and Sastre, 2001). Here we show that the *Drosophila* SRF homolog, *blistered* (*bs*), can be implicated in the regulation of a fiber-specific actin, known as *Act88F*.

This study will broaden our understanding of the roles of factors such as SRF and *bs* in muscle fiber diversification and further provide insight into the mechanisms that lead to fiber specific differentiation.

METHODS

Flies

Fly stocks were obtained from the Bloomington *Drosophila* Stock Center or Vienna *Drosophila* RNAi Center (VDRC) and maintained on Fisher-Scientific Jazz Mix medium. The *1151-Gal4* muscle specific driver was utilized during the RNAi knockdown experiments to drive expression of *Act88F* in flight muscles at the adult stage. Equal numbers of virgin females (*1151;Act88F-lacZ)* and males (*UAS-bs RNAi*) were crossed and incubated at 25°C until white pupae formed. Crosses were then placed in the 29°C to induce tissue-specific effects in the flight muscles of the developing pupa (Bryantsev *et al*. 2012). The RNAi-inducible fly line: 100609 (bs, VDRC) was used in this study. The progeny of the crosses were collected and prepared for tissue analysis or reporter assay studies (Liquid beta-galactosidase assay).

Tissue Analysis

Cryosections were prepared and analyzed as described by Jaramillo *et al*., 2009. Newly enclosed flies were embedding into Tissue Tek (OTC) Freezing Medium (Sakura). Sections were cut at 10 μm thickness at -18°C using Triangle Biomedical Services Minotome Plus. Sections were fixed in 10% formaldehyde (3.7% v/v) in PBS for 8 minutes before being washed in PBTx (1x PBS, 0.2% v/v Triton-X100). Phalloidin was obtained from Molecular Probes and diluted to 1:400 in PBTx and 1% BSA (Bovine serum albumin 1% w/v) and used to identify myofibrillar structures. For immunofluorescent detection, Alexa conjugated

(Molecular Probes) secondary antibodies, Alexa 488 and Alexa 568, were diluted to 1:400 and mixed with DAPI (Sigma) at 1uL/mL. Confocal images obtained with a Zeiss LSM-780 were assembled and processed using Adobe Photoshop.

Cell Culture

Drosophila S2 cells were maintained at 25°C in standard Schneider medium (Gibco) containing 10% fetal bovine serum (Atlanta Biologics). Cell transfection assays were performed with Trans IT-2020 (Mirus Bio) according to manufacturer's instructions, which call for 3uL transfection reagent per 1ug of transfected DNA. For normalization purposes, an empty vector was used as a negative control in co-transfections with *bs, Act88F* and *exd/hth.* Cells were transfected with a 1:1 ratio of activator plasmid (*pAW-bsRA*, *pAW-exd*, *pAW-hth*) to reporter plasmid (*pCHAB-Act88F(1kb)nLacZ*, *pCHAB-Act88F(Reg1)nLacZ*) to equal 1ug of total DNA. Following incubation at 25°C, cells were lysed 24 hours after transfection and analyzed for Beta-galactosidase expression (Liquid beta-galactosidase assay) or prepared for RNA extraction (Qiagen RNeasy kit).

Liquid Beta-galactosidase Assay

Individual frozen newly eclosed adults were homogenized in 100 uL PBTx and briefly centrifuged. Aliquots of lysates were mixed with beta-Galactosidase Assay Reagent (Thermo Scientific) in a 96-well plate, and placed in a multi-well plate reader at 37C. Sample absorbance was taken at 405 nm at 2 minute time intervals for a total of 22 minutes. The average absorbance was taken from 3 separate time points to calculate B-gal activity per fly. Data is

shown as a percentage relative to positive control flies, which were analyzed at the same time. The same procedural steps were used to assess B-gal activity in transfected cells, however cells were instead shaken at 350 rpm for a total of 20 minutes in order to lyse cellular membranes.

Expression Analysis

Flight muscles were extracted from newly eclosed (less than a day old) wild-type adult flies in addition to flies containing the *bs* genetic knockdown. Flies were covered in Tissue Tek (OTC) medium to separate thoraces. Thoraces were then transferred to 1% sucrose solution; muscles were extracted and transferred to a lysis buffer supplied by the Qiagen RNeasy Mini extraction kit. RNA was then extracted according to the Qiagen RNeasy Mini protocol. Following RNA extraction, cDNA was synthesized using Invitrogen Superscript II Reverse Transcriptase from a reaction mixture containing 100 ng extracted RNA, 10mM dNTPs, 5x First Strand Buffer, 0.1 mM DTT, and random hexamer primers (Roche). Diluted cDNA was used as a template for PCR analysis with Pfx Polymerase (Invitrogen) and the following gene specific primers were used:

bs_all:

5'TCGACGACAGCGTAGACAAC3'

5'TGTAGCGACGCAGCTTATTG3'

bs_RB:

5'ACTACAGCCTCGAGCAGAGC3'

5’AATCCTAGCCAGAAGCCTAGC3’

bs_RA:

5’CCATGCCAGCATTGAACTATC3’

5’GCAGCGGAGTAGACGTACTTG3’

RT-PCR cycles were repeated 30-40 times per experiment. cDNA template dilutions were adjusted to that of the loading control (WT) and amplified equally across all samples. Final amplification products were visualized on a 2% agarose gel.

RT-qPCR analysis was carried out using the Applied Biosystems 7000 Real-Time PCR System. Both wild-type and *bs* KD cDNA samples were combined in a reaction mixture containing 2x SYBR Green Master Mix (Bio-Rad) and *Act88F* primers below. All samples were run in the presence of normalized *Act88F* standards and mean transcript levels analyzed after each replicate run. The same procedure was used to measure 18S rRNA in both samples.

Act88F:

5’AGCTCTTCAAAGGCAGCAAC3’

5’ATTGTTGTGCGATGGGTTC3’

18S:

5’TTCATGCTTGGGATTGTGAA3’

5’GGGACGTAATCAATGCGAGT3’

Molecular Cloning

The pBluescript II-DSRF plasmid encoding the *bs-RA* isoform was obtained from Markus Affolter (University of Basel, Switzerland). Gateway Recombination (Invitrogen) was utilized to insert the *bs-RA* sequence into a pAW destination vector. DNA encoding *bs-RA* was initially PCR amplified from the pBluescript II-DSRF plasmid using the following primers. Bold letters denote the attB sequences needed to recombine the PCR fragment into the pDONR(221) entry vector. Additional constructs, including pAW-exd, pAW-hth, and pCHAB-Act88FnLacZ, were also utilized (Bryantsev et al. 2012).

bs_RA:

5’**GGGGACCACTTTGTACAAGAAAGCTGGGT**GCTAATCGAACTCCTGTTT3’

5’**GGGGACAAGTTTGTACAAAAAAGCAGGCT**ACTTTTACCTCAGAATGGAT3’

Bioinformatics

Two identified regions within the *Act88F* enhancer, bBS1 and bBS2, containing similarities to the classical CArG consensus sequence were compared across all 12 *Drosophila* species using a BLAST search through the FlyBase database (Flybase.org/blast). BLAST hits were aligned against sequences corresponding to bBS1 and bBS2 in *D. melanogaster*.

Electrophoretic mobility Shift Assay (EMSA)

Drosophila S2 cells were transfected with *pAW-bsRA* and nuclear extract was prepared (Andrews and Faller, 1991). Binding reactions were then carried out and analyzed on a 5% polyacrylamide gel (LightShift Chemiluminescent EMSA Kit, Thermo Scientific). The following biotin labeled and unlabeled double-stranded oligonucleotide sequences were used for binding reactions. Bolded region indicates sequence corresponding to the CArG box.

bBS1_biotin:

5'[Btn]TCTGAAAACT**GCTTATATGG**ATCGATTGTT3'

5' [Btn]AACAATCGAT**CCATATAAGC**AGTTTTCAGA3'

bBS1:

5' TCTGAAAACT**GCTTATATGG**ATCGATTGTT3'

5' AACAATCGAT**CCATATAAGC**AGTTTTCAGA3'

bBS2_biotin:

5'[Btn]CCTTGATGTT**GATTTATAGG**TGCCGCTCTG3'

5'[Btn]CAGAGCGGCA**CCTATAAATC**AACATCAAGG3'

bBS2:

5'CCTTGATGTT**GATTTATAGG**TGCCGCTCTG3'

5'CAGAGCGGCA**CCTATAAATC**AACATCAAGG3'

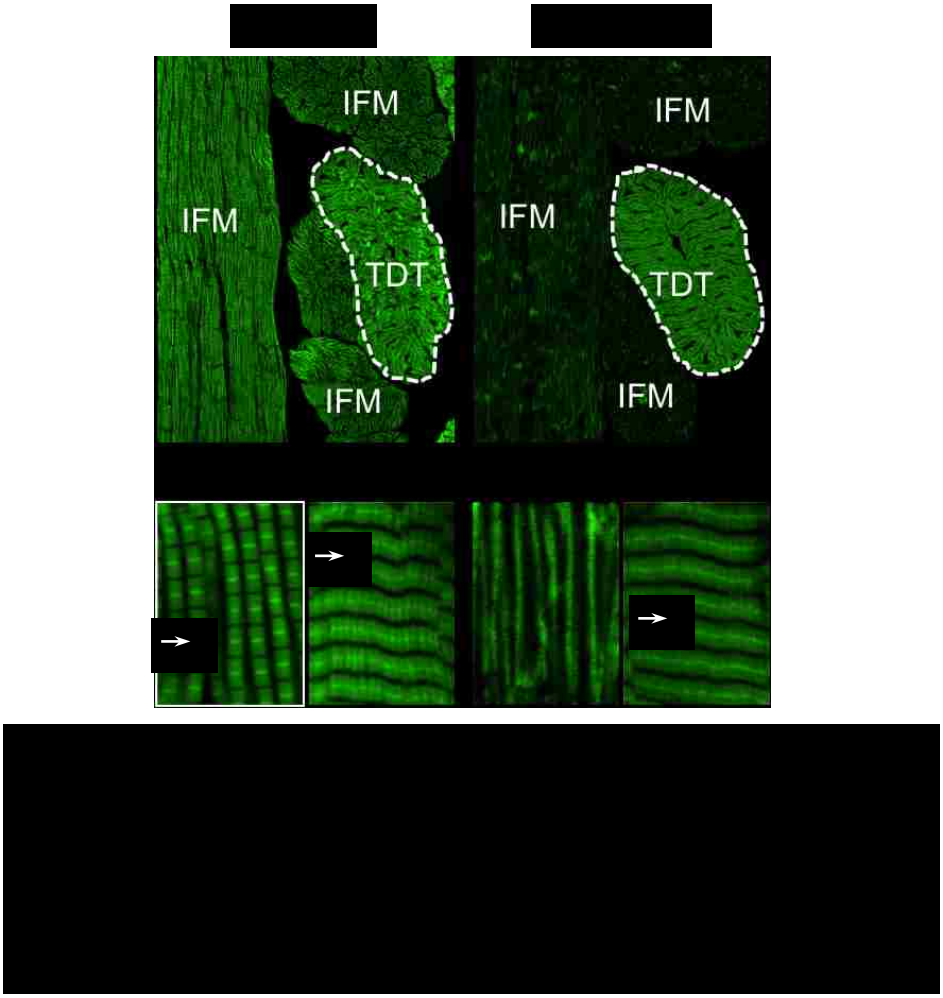
RNA-Seq Analysis

RNA was extracted from flight muscles of *bs* knockdown and wild-type flies and sent to Columbia Genome Center for processing (systemsbiology.columbia.edu). For our purposes single-end reads were used and generated approximately 30 million reads per sample. The data were then analyzed using DNANexus cloud computing (dnanexus.com) to identify differentially expressed genes.

RESULTS

Specified phenotype of the *bs* knockdown

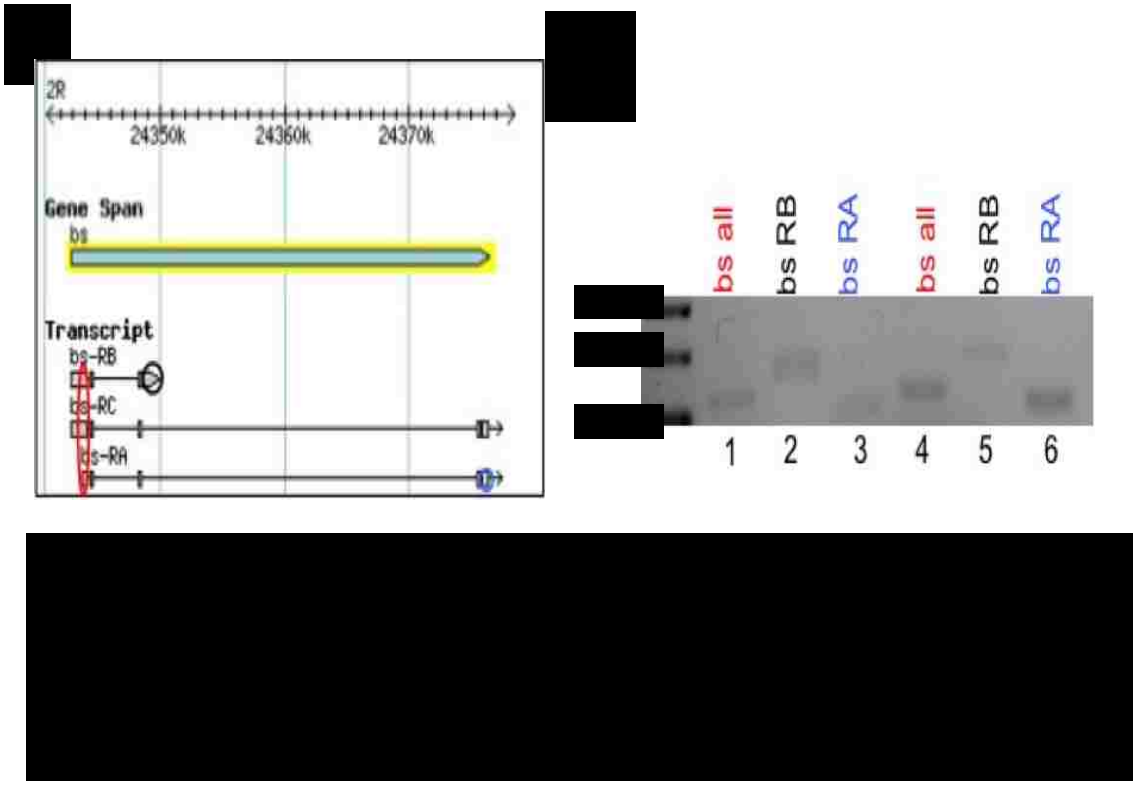
Identification of potential regulators of muscle differentiation in *Drosophila* flight muscles was carried out in order to obtain a better understanding of the factors that contribute to the process. Expression of each gene identified to be involved in muscle differentiation was then knocked down. Muscles of the adult thorax and flight capability of knockdown flies were assessed for abnormalities. RNAi-based knockdown (KD) of *bs* in muscles, achieved through use of the flight muscle specific driver *Actin88F* resulted in a unique muscle fiber phenotype that showed structural and morphological changes compared to wild-type. In contrast to wild-type flight muscles, the flight muscles of the *bs* knockdown contain immature and disorganized myofibrils, while the myofibrils of jump muscles, as well as other somatic muscles, were unaffected and remained intact (Figure 1).



Transcript isoforms of *bs*, alone, do not account for flight muscle specific phenotype

Next, we investigated whether this specific phenotype was due to the differential expression of *bs* isoforms. There are three annotated *bs* transcript isoforms, *bs-RB*, *bs-RA*, and *bs-RC* (Figure 2A). Given that transcripts corresponding to the RA and RC isoforms are nearly identical and lack discrepancies in their protein coding sequence, they were considered as the same transcript. RA/RC and RB, on the other hand, encode potentially different protein isoforms. Therefore, *bs-RA* was tested along with *bs-RB* to determine

possible roles played in production of the *bs*KD phenotype. First, the presence of *bs* isoforms in both flight muscles and jump muscles of wild-type flies was tested (Figure 2B). cDNA from each isolated muscle along with primers designed to detect specific regions of selected bs isoforms were used for RT-PCR. Both *bs-RB* and *bs-RA/RC* are present in jump and flight muscles. These results indicate that there may be other factors that influence the regulation of either isoform within the flight muscles.



In order to analyze mechanisms of flight muscle specification in the *bs* knockdown phenotypes, RNAi constructs specifically targeting either the RA/RC or RB transcript isoforms were created and analyzed (data not shown). The initial *bs-RA/RC* knockdown was flight muscle specific; however, the RNAi

construct targeting *bs-RB* produced unexpected results. Although *bs-RB* RNAi worked to produce a knockdown of *bs* in trachea, a tissue known to express *bs* at high concentrations, it failed to induce a phenotype in muscles even after using two independent muscle-specific drivers. These results indicate that *bs-RB*, alone is not involved in regulation within the flight muscles and only *bs-RA* appears to be involved in flight muscle differentiation.

Muscle specific *Actin88F* is responsive to *bs* knockdown

Reduced expression of *bs* led to alterations in the myogenic process for only a subset of muscle fibers. Myofibers of flight muscles were immature and disorganized when *bs* was not present, whereas other surrounding muscles were not affected. While the physical characteristics that resulted from knockdown of *bs* were immediately apparent, differences at the molecular had yet to be investigated. To assist in the identification of additional genes that may be involved in muscle differentiation RNA-seq was utilized. RNA-seq relies on next-generation sequencing technologies to provide a measurement of transcript expression and thus allows for comprehensive analysis of gene expression. RNA was extracted from flight muscles of *bs* knockdown and wild-type flies and sent to Columbia Genome Center for processing. Whole-transcriptome normalized data revealed expression differences between flight muscles of wild-type and that of the *bs* knockdown (Figure 3). Although not many genes were statistically up or down-regulated, one of the few that had a significant change in expression was the gene *Act88F*. This gene was of interest for several reasons. First, the expression differences between wild-type flight muscles and those of

the *bs*KD were rather large. For example, *Act88F* transcripts were expressed in high amount in wild-type but decreased drastically in the flight muscle where *bs* expression has been reduced. Secondly, previous studies identify *Act88F* as a gene expressed specifically in the flight muscles, which are the muscles affected by knockdown of *bs*. Given these data *Act88F* was hypothesized as a potential target of the transcription factor *bs*. Moreover, it was predicted that the enhancer region of *Act88F* would respond to presence of *bs* through binding of the transcription factor at the enhancer to regulate expression of the gene.

gene	expression in control muscle (FPKM)	expression in bs KD muscle (FPKM)	log[2] fold change	notes on gene
DOWN-REGULATED				
Ag5r	95	3	-5.0	secreted protein; unknown function
CG1678	1508	48	-5.0	function unknown
CG3837	40	3	-3.6	secreted insulin receptor; regulation of growth
CG43679	832	80	-3.4	function unknown
CG16826	216	25	-3.1	function unknown
Act87E	1080	225	-2.3	muscle-specific actin
Act88F	22090	5210	-2.1	muscle-specific actin
UP-REGULATED				
nrv2	8	70	3.1	cation transmembrane transporter
CG11880	2	17	3.4	choline transporter-like
Mbs	9	96	3.5	myosin phosphatase
Arc1	6	100	4.1	cytoskeleton associated protein
CG14688	13	230	4.1	phytanoyl-CoA dioxygenase; lipid metabolism

[redacted]

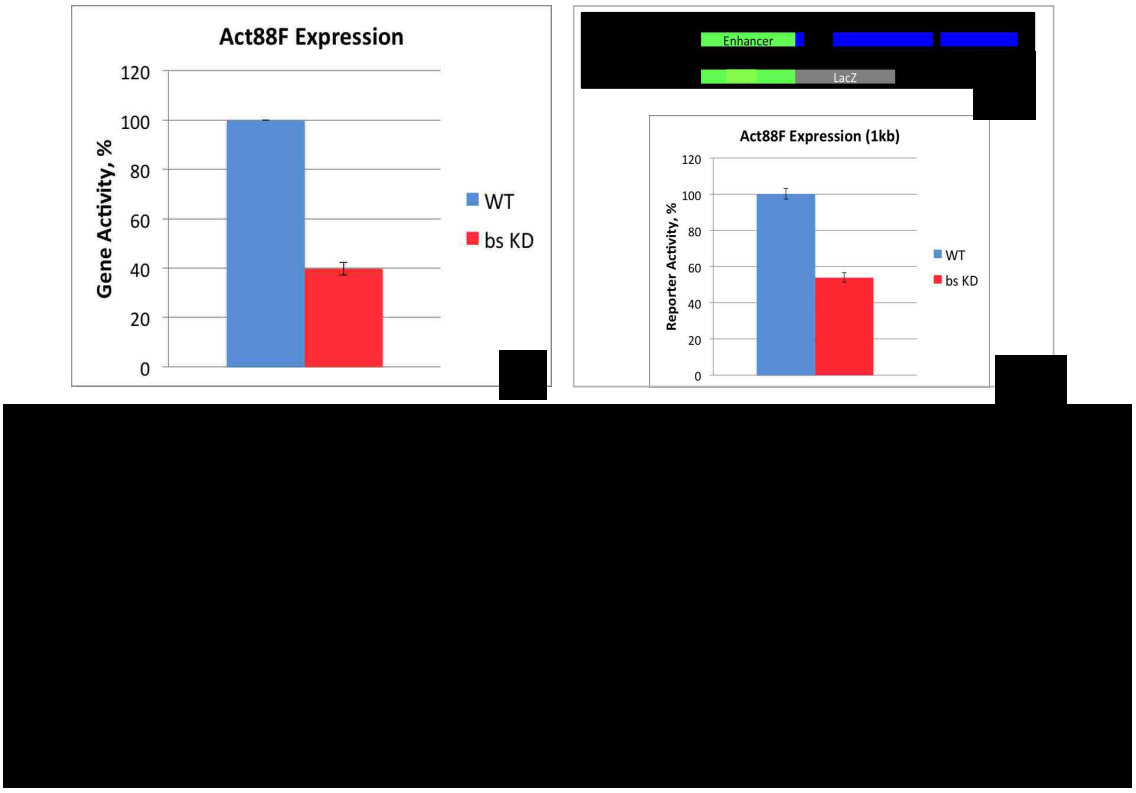
Down-regulation of *bs* affects expression of *Act88F in vivo*

Next, the role of *bs* in the differentiation of flight muscles was investigated. First, *Act88F* expression was monitored in response to knockdown of *bs* to understand if this flight muscle specific gene is a target of *bs*. cDNA was

generated from RNA extracted from wild-type flight muscles and *bs* depleted flight muscles. RT-qPCR was used to quantify expression of endogenous *Act88F* in both samples (Figure 4A). Compared to wild-type, there was a significant decrease in the expression of *Act88F* upon *bs* KD.

To further assess the effects of down-regulation of *bs* in all muscles, a reporter was created to probe for the transcriptional regulation of *Act88F* when *bs* is knocked down in flies. This was accomplished by the fusion of a 1kb upstream region of *Act88F* to the *LacZ* reporter gene (Figure 4B). In this transgenic construct, the cloned enhancer region was sufficient to direct expression of beta-galactosidase in flight muscles, which indicated that the 1kb enhancer contained all the regulatory elements controlling *Act88F* expression. Furthermore, flies containing *Act88F(1kb)LacZ* were compared between control and *bs* KD flies. Beta-galactosidase, produced by the expression of the *Act88F(1kb)LacZ* reporter, was then detected in fly lysates by a quantitative beta-galactosidase activity assay.

Results show an approximately 50% reduction in reporter activity when *bs* is downregulated (Figure 4C), that correlates well with the expression difference of the endogenous gene upon *bs* KD. Overall, *Act88F* activity is affected upon *bs* KD, thus *Act88F* is a possible target of *bs,* with a potential binding site located within the *Act88F* enhancer region.

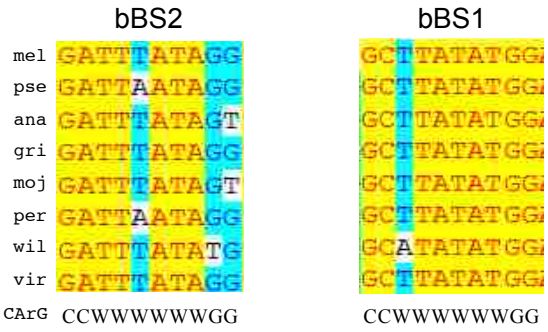


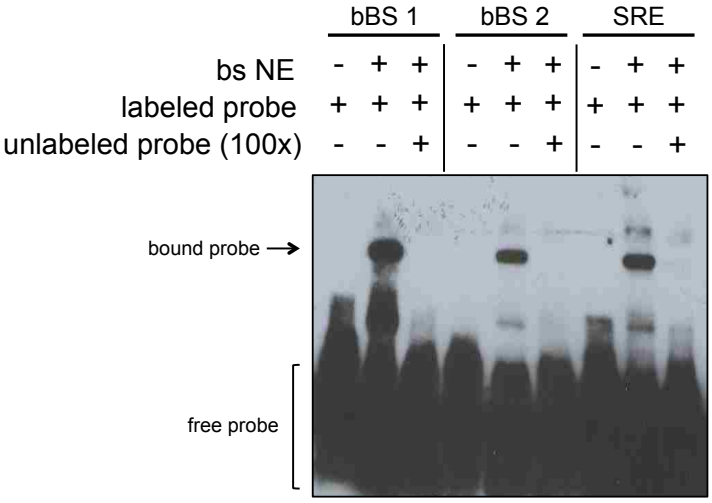
bs physically interacts with *Act88F* at bBS1 and bBS2 *in vitro*

Given that the genetic expression of *Act88F* is dependent on the *bs* gene, we wanted to know whether the bs protein is a direct regulator of *Act88F* expression. Due to previous findings that show mammalian SRF binds the upstream region of actin genes at the CArG site (Miano, 2003), two binding sites within the *Act88F* enhancer containing similarities to the consensus sequence were chosen for further investigation (Figure 5A). These sites identified as bBS1 and bBS2 are conserved across all *Drosophila* species, with bBS1 harboring slightly more similarities to the CArG consensus sequence than bBS2 (Figure 5B). The highlighted blue and yellow areas indicate regions of absolute conservation between *Drosophila* species. To determine the capability of *bs* to

bind to the enhancer region of *Act88F* at each identified site an Electrophoretic mobility Shift Assay (EMSA) was utilized (Figure 5C).

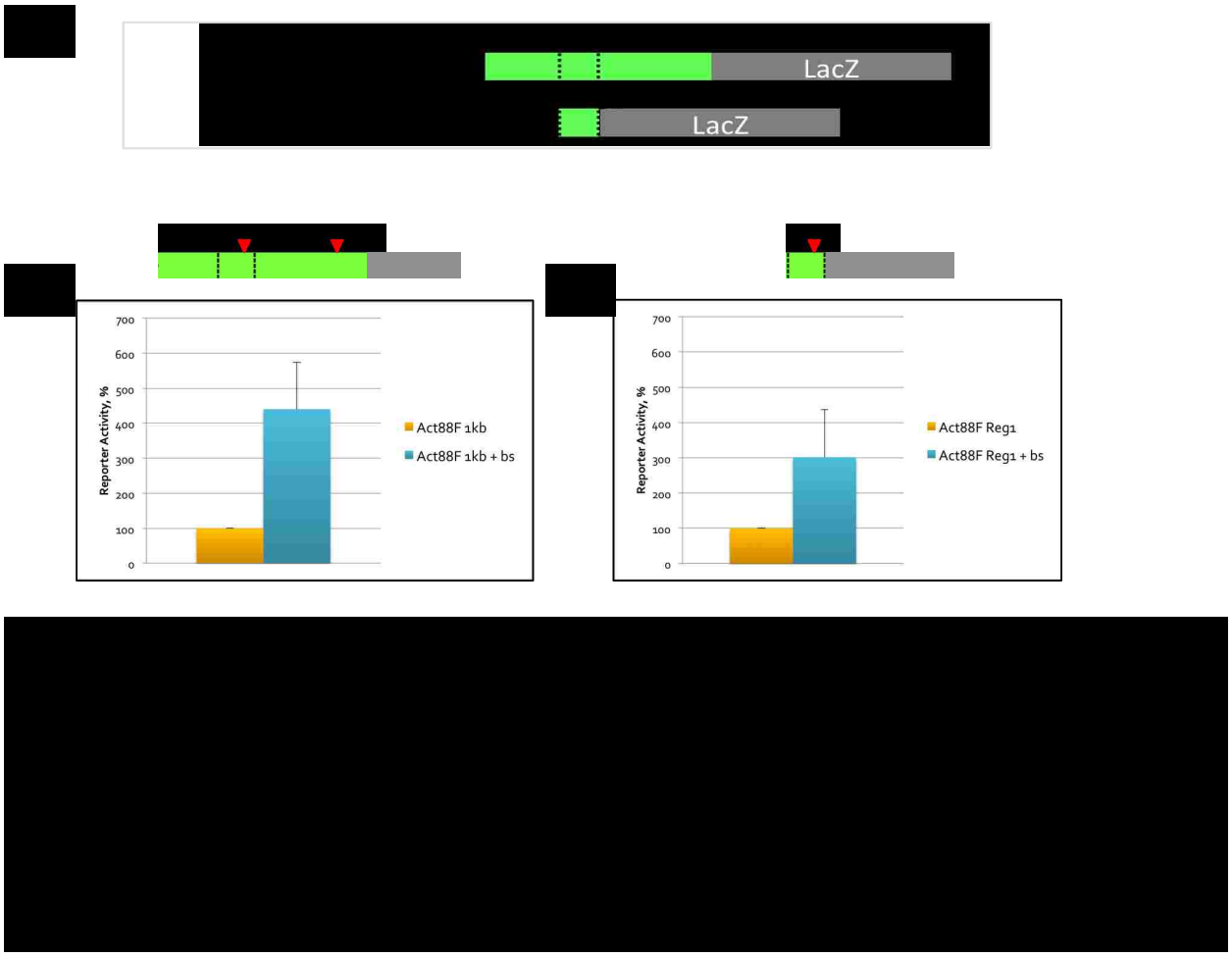
The EMSA was preformed using oligonucleotides corresponding to bBS1 (Figure 5C, Lanes 1-3), bBS2 (Figure 5C, Lanes 4-6), in addition to a positive control (SRF) (Figure 5C, Lanes7-9) whose sequence is identical to the classical CArG site. As a source of bs protein, nuclear extract from *Drosophila* S2 cells that had been transfected with the *bsRA* expression plasmid were used. When nuclear extract was combined with either of the labeled oligonucleotides, a more slowly migrating band was observed corresponding to the complex between *bs* and each of the tested sites (Figure 5C, Lanes 2, 5, and 8). Subsequent addition of the same unlabeled oligonucleotides as a competitor was successful in eliminating the previously seen band (Figure 5C, Lanes 3, 6, and 9). Here results show *bs* physically interacts with the *Act88F* enhancer at bBS1 and bBS2. Based on the similarity of bBS1 to the classical consensus sequence and the intensity of the band visualized binding of *bs* at bBS1 is hypothesized to have a stronger binding affinity for the *Act88F* enhancer.


bBS2
bBS1
mel GATTTATAGG GCTTATATGG
pse GATTAATAGG GCTTATATGG
ana GATTTATAGT GCTTATATGG
gri GATTTATAGG GCTTATATGG
moj GATTTATAGT GCTTATATGG
per GATTAATAGG GCTTATATGG
wil GATTTATATG GCATATATGG
vir GATTTATAGG GCTTATATGG
CArG CCWWWWWWGG CCWWWWWWGG


bBS 1
bBS 2
SRE
bs NE - + + - + + - + +
labeled probe + + + + + + + + +
unlabeled probe (100x) - - + - - + - - +
bound probe
free probe

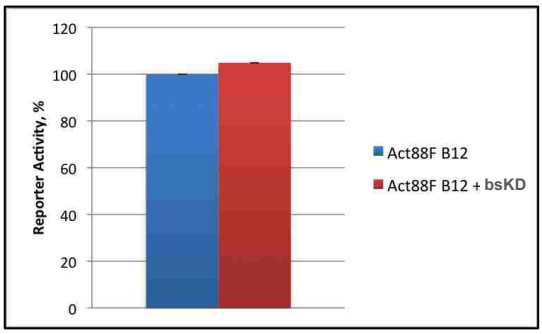
Binding of *bs* at bBS1 and bBS2 activates the *Act88F* enhancer *in vivo*

To further validate the specific interaction between the *Act88F* enhancer and the two *bs* binding sites, bBS1 and bBS2, the function of each site in the native cellular environment of *Drosophila* S2 cells was assessed. Such analyses utilized,the *Act88F* 1kb reporter previously tested in flies, as well as a Reg1 reporter, which is composed of a 52 base pair truncation of the *Act88F* 1kb enhancer (Figure 6A). Closer inspection of both constructs revealed the 1kb reporter includes both *bs* binding sites whereas the Reg1 reporter only contains the binding site for bBS2. Cells were co-transfected with either of the *Act88F* reporter constructs in addition to a expression construct containing the *bsRA* isoform to determine if *bs* activates the *Act88F* enhancer *in vivo*. Results from transfection assays reveal cells that express the 1kb reporter in addition to *bs*, show an increase in reporter activity relative to cells expressing the reporter alone (Figure 6B). Similarly, cells that express the Reg1 reporter and *bs* also show an increase in reporter activity (Figure 6C), but activation is not as efficient as that seen when using the full length reporter. Overall, although both *bs* binding sites are functional *in vivo*, when bBS1 is not available for *bs* to bind, reporter activity is not as robust. These results therefore indicate presence of bBS1 is important for activation of the *Act88F* enhancer *in vivo*.



In addition to such studies that indicate the availability of bBS1 is important for *Act88F* activation, the effect of additional enhancer variants on expression of *Act88F* was also tested by means of the B12 reporter, which includes bBS2 but excludes bBS1 (Figure 7A). For these analyses, transgenic flies harboring the *Act88F* B12 reporter were crossed to lines expressing the *bs* knockdown. Progeny containing *Act88F(B12)LacZ* were compared to those expressing the reporter in addition to a knockdown of *bs*. Beta-galactosidase, produced by the expression of the *Act88F(B12)LacZ* reporter, was then detected in fly lysates by a quantitative beta-galactosidase activity assay. Results show

there was no difference in reporter activity in flies that expressed both the reporter and a knockdown of *bs* (Figure 7B). Comparing these results to data taken from expression of the entire *Act88F* 1kb reporter in flies (Figure 4A), where there is a decline in reporter activity, further highlights the sensitivity of *Act88F* expression when bBS1 is not present. It is suggested that the B12 reporter is not affected by absence of *bs*, due to the elimination of bBS1, therefore it is likely that *bs* binds at bBS1.



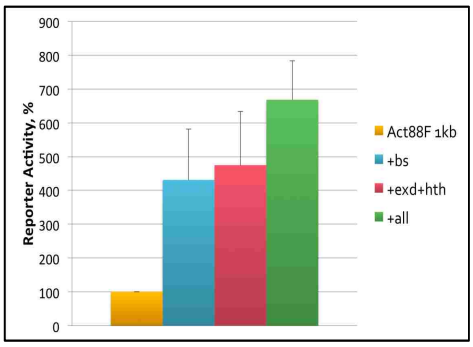
[redacted]

_bs_ and _exd/hth_ initially compete for binding within Reg1

Next, the roles played by the evolutionarily conserved transcription factors *exd* and *hth* were tested. These pioneer factors work in conjunction with other fiber identity genes, such as *Act88F* to determine muscle fiber fate.

Additionally, these transcription factors are known to bind together to the Reg1 sequence within the *Act88F* enhancer (Bryantsev et al., 2012), but their association with *bs* has not been investigated. To further elucidate the potential collaboration between *bs* and these factors at the *Act88F* enhancer, additional manipulation of *Drosophila* S2 cells was carried out. Cells were transfected with *Act88F(1kb)LacZ* or *Act88F(Reg1)LacZ* in addition to different combinations of *bs*, *exd* and *hth*. Transfection assay results indicated cells that express *Act88F(1kb)LacZ* along with *bs* and *exd/hth*, showed an added increase in reporter activity (Figure 8A) compared to cells expressing the reporter and bs alone. On the other hand, those cells that express *Act88F(Reg1)LacZ* in conjunction with all other factors did not reciprocate this trend, as there appeared to be no boost in reporter activity compared to cells that express the Reg1 reporter alone (Figure 8B). Given that both *exd/hth* and *bs* bind within Reg1, these results suggest *exd/hth* and *bs* compete within Reg1 of the *Act88F* enhancer for binding and therefore it is likely *bs* initially binds at bBS1 instead of bBS2.

[redacted]



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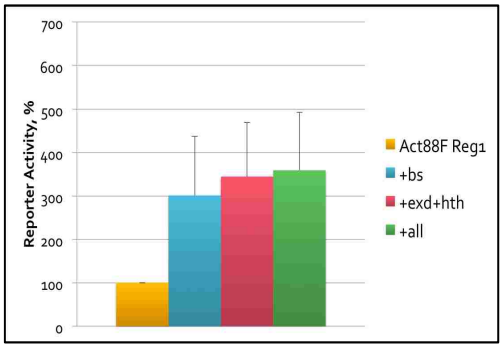

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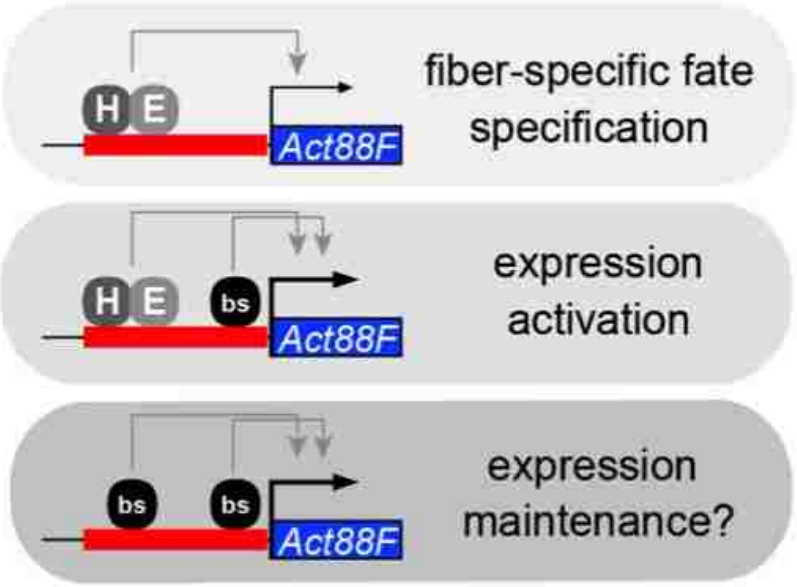
Proposed mechanism of fiber specificity

Based on data presented here as well as results from previous studies, the following model for fiber specificity is proposed (Figure 9A). Early in the process, the fiber-specific factors, Exd/Hth, are responsible for the specification of *Act88F* in flight muscles. During this transient stage, Bs binds with the help of Exd and Hth who open the chromatin and further make the DNA accessible, thus causing an increase in *Act88F* transcriptional activity. Lastly, it is proposed that late in muscle development, Exd/Hth leave and allow an additional *bs* transcription factor to bind allowing the flight muscle to maintain a robust level of *Act88F*.



DISCUSSION

The mechanism by which muscle fiber differentiation occurs is a relatively uncharacterized process in the developing adult fruit fly. In previous work, the control over flight muscle fate was attributed to a pair of fate-determining genes, *exd* and *hth* (Bryantsev et al., 2012). However, our findings implicate the gene *blistered* in the process responsible for terminal differentiation of flight muscles. Here we show *bs* works in conjunction with the fiber-specific factors, *exd* and *hth*, to specify and later regulate the expression of *Act88F*, a flight muscle specific gene. Removal of *bs* results in a flight muscle specific phenotype characterized by the aberrant appearance of myofibrils and reduced expression of muscle specific actin, solely within the flight muscles. Although muscle physiology has

been well characterized (Burton, 2002; Pette and Staron, 1997; Baskin et al., 2015; Agudelo et al., 2014), not much has been known about the developmental process that determines the formation of different types of muscle fibers. Our studies further help fill this void by working to identify the mechanism by which *bs* contributes to muscle fiber differentiation.

In our research we have identified *bs* as being an important factor for the formation of a single group of muscle fibers. Fibrillar muscle fibers, which make up the flight muscles, are the only fibers that seem to be affected upon elimination of *bs*. The restricted changes in morphological and structural appearance of flight muscle myofibers identify *bs* as playing an important and selective role in the development and differentiation of muscles found within the adult thorax of *Drosophila*. Research conducted here has shown that the selectivity of *bs* is further attributed to its ability to bind flight muscle specific *Act88F*. Additional investigation into the presence of *bs*, conducted through overexpression experiments, would further elucidate the response of *Act88F* in both a native and ectopic environment.

The other major muscle found in the adult thorax, the jump muscle, was found to be unaffected by down regulation of *bs*. Jump muscles composed of tubular type fibers, unlike the fibrillar fibers characteristic of flight muscles, and instead express the muscle actin gene *Act57B* (Fyrberg et al., 1983). Moreover, the pioneer factors known as *exd* and *hth*, that are known to promote flight muscle identity, are not expressed in jump muscles (Bryantsev et al., 2012). Given that we have provided a direct mechanistic link between *exd*/*hth*, *bs*, and

the flight muscle specific *Act88F*, further explains why jump muscles are not affected by elimination of *bs*. Jump muscles simply lack the cooperation between these factors and therefore appear normal upon knockdown of *bs*.

Furthermore, I have identified two regulatory regions within the *Act88F* enhancer that contain conserved binding sequences for *bs*. Through molecular analysis, we were able to establish *bs* affects expression of *Act88F* through interaction at these specific regions within the enhancer. Given *Act88F* expression is dependent on the activities the two fiber-identity factors, *exd* and *hth* (Brayantsev et al., 2012), we were able to establish a connection between these factors and *bs*. Our studies specifically indicate cooperation between all three factors to promote sustained *Act88F* expression in flight muscles during development of the adult musculature. Future investigation into the participation of additional pioneer factors might be interesting and would further our understanding of the mechanisms behind fight muscle specificity.

Pioneer factors have long been known to play an important role in the regulation of many muscle genes. Pbx and Meis, the vertebrate orthologs to Exd and Hth have been shown to work in conjunction with MyoD, a transcription factor that regulates skeletal muscle development (Maves et al., 2007; Tapscott 2005). In addition, recent research has shown MyoD is regulated by another early pioneer factor, known as NFAT1, to promote slow to fast twitch fiber conversion (Ethers et al., 2014). Based on previous evidence and as seen in results presented here, the interaction of pioneer factors early on in myogenesis is essential for control of fiber type specification.

Since the presence of different fiber types is a universal feature of all organisms, our findings may shed light on the process of muscle fiber specification as it occurs in vertebrate myogenesis. In mice, SRF is known to regulate actin and therefore affect skeletal muscle differentiation (Miano et al., 2007) as similarly seen in our experiments with *bs* and *Act88F*. In zebrafish, the fate of fast twitch muscle fibers is controlled by PBX and MEIS, which are orthologs to *exd* and *hth* in *Drosophila* (Maves et al., 2007). The appearance of such factors across species suggests the mechanism for fiber specification is well conserved. Insight into this conserved process will promote identification of additional factors that may participate in formation of other types of muscle fibers, thus leading to a more robust understanding of muscle differentiation.

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